Induction of Apoptotic Cell Death in HL-60 Cells by Acteoside, A Phenylpropanoid Glycoside

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Acteoside, a phenylpropanoid glycoside with anti-oxidative activity, induced cell death in promyelocytic leukemia HL-60 cells with an IC_{50} value of 26.7 μM. Analysis of extracted DNA on agarose gel electrophoresis revealed that acteoside induced the internucleosomal breakdown of chromatin DNA characteristic of apoptosis. Apoptosis-specific DNA fragmentation was clearly detectable 4 h after treatment with acteoside and was indepedent of the cell cycle phase. These data indicate that acteoside induces apoptosis in HL-60 cells.

Key words   acteoside; apoptosis; HL-60; phenylpropanoid glycoside

Phenylpropanoid compounds are glycosides of phenylethyl alcohol esterified by a cinnamic acid molecule (*e.g.*, caffeic acid, ferulic, or *p*-coumaric acid) and widely distributed in plants such as *Phlomis armeniaca*,[1] *Scutellaria salviifolia*,[1] *Orobanche ramosa*,[2] *Lippia dulcin*,[3] *Cistanche tubulosa*[4] *etc.* Several phenylpropanoid glycosides have been shown to have anti-oxidative, anti-bacterial and anti-fungal activities, as well as the inhibitory activities of cyclic-AMP phosphodiesterase, aldose reductase and protein kinase C. Acteoside, one such phenylpropanoid glycoside, also shows such biological activities as protective action on KCN-induced anoxia,[5] immunosuppressive activity,[6] antihypertensive activity[7] and anti-tumor activity.[8] In our previous study we also demonstrated that acteoside expresses cytotoxic activity against some cancer cells (dRLh-84 (rat hepatoma), S-180 (sarcoma), P-388/D1 (mouse lymphoid neoplasma), HL-60 (human promyelocytic lymphoma) (unpublished data)), but not against primary-cultured rat hepatocytes.[1] In addition, study of the structure–activity relationship showed that *o*-dihydroxy aromatic systems of phenylpropanoid glycosides were necessary for their cytotoxic activity. Recently, cell death has attracted attention comparable to that focused on cell growth because cell death is regarded as active self destruction to control normal embryogenesis, development of the immune system, elimination of virus-infected cells and the maintenance of tissue homeostasis[9—11] in addition to a degenerative phenomenon produced by injury. Apoptosis, an alternative mode of cell death to necrosis, is well known to be a process by which organisms eliminate damaged, precancerous or excessive cells. However, increasing evidence suggests that the two forms of cell death are similar in characteristics concerning early signal transduction and that the intensity of insult appears to determine which type of cell death is elicited.[12] In this study, we investigated the anti-cancer activity of acteoside using human promyelocytic leukemia HL-60 cells, which are widely used in the study of apoptosis, and found that acteoside induced typical apoptosis in HL-60 cells.

MATERIALS AND METHODS

Materials   Acteoside was isolated from the air-dried aerial parts of *Phlomis armeniaca* WILLD, *Scutellaria salviifo-lia* BENTHAM.

Reagents   RPMI1640 and fetal calf serum (FCS) were purchased from Irvine Scientific Co. (Santa Ana, CA). Proteinase K, RNase A, penicillin, streptomycin and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells   HL-60 (human promyelocytic leukemia) cells were provided from Japan Cancer Research Resource Bank and were cultured in RPMI1640 medium supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin.

Cytotoxicity Assay   HL-60 cells were seeded into a 96-multi-well plate at a concentration of 6×10^4 cells/ml. Acteoside was dissolved in medium, sterilized through a 0.22 μm membrane filter, and then added into the cell culture at appropriate concentrations, followed by incubation in 5% CO_2 in air for 48 h. The cytotoxic activity was determined by measuring the surviving cell number using the trypan blue dye exclusion method.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis   HL-60 cells (6×10^6 cells/10 ml/dish) exposed to 50 μM acteoside for 6 h were collected into a tube and then washed with phosphate-buffered saline (PBS). The cells were incubated for 10 min in 500 μl of lysis buffer (20 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.2% Triton X-100) at room temperature and centrifuged at $10000 \times g$ 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. of chloroform/phenol (1 : 1), precipitated from the aqueous phase with 1 vol. of isopropanol and 500 mM NaCl at −20 °C overnight, and collected by centrifugation at $14000 \times g$ for 30 min at 0 °C. The pellet was resuspended in 70% ethanol and centrifuged at $14000 \times g$ for 10 min at 0 °C. The pellet was dried under reduced pressure and incubated in 25 μl of 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 applied to agarose gel electrophoresis after the addition of a loading buffer. Horizontal electrophoresis was performed for 1 h at 80 V in 1.5% agarose gel with TBE (×0.5) as a running buffer. After treatment of the gel with 0.5 μg/ml of ethidium bromide for 10 min, DNA was visualized by UV illumination.
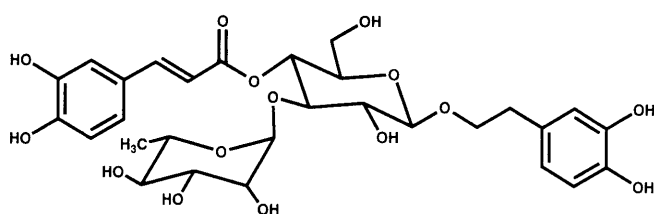
Flow Cytometric Analysis   HL-60 cells (6×10^4 cells/ml) exposed to 50 μM acteoside were harvested by cen-

trifugation 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 RNase A solution (0.112 mg/ml trisodium citrate solution) at 37 °C for 30 min. Cells were harvested by centrifugation at 1500 rpm for 5 min and stained with 250 μl of nuclear staining solution (10 mg propidium iodide, 0.1 mg trisodium citrate, and 0.03 ml Triton X-100 were dissolved in 100 ml H_2O) at room temperature for 30 min in the dark. After adding 750 μl PBS, the DNA content in each cell cycle phase was determined by FACScan (Beckton Dickinson).

RESULTS

Acteoside is a phenylpropanoid glycoside, the structure of which is illustrated in Fig. 1. We first determined whether acteoside exhibits cytotoxic activity against HL-60 cells, human promyelocytic leukemia cells. Figure 2 showed that acteoside elicited cell death in HL-60 cells and that its IC_{50} was 26.7 μg/ml. Light microscopic observation revealed that acteoside induced morphological changes characteristic of apoptosis such as the disappearance of microvilli, cell shrinkage, and chromatin condensation (data not shown). In general, apoptosis is well known to be associated with the fragmentation of DNA into internucleosomal fragments of 180-200 base pair. DNA extracted from HL-60 cells exposed for 6 h to 50 μg/ml acteoside or 2.5 μg/ml actinomycin D, an inhibitor of mRNA synthesis and a well-known apoptosis inducer, was then subjected to agarose gel electrophoresis. As shown in Fig. 3, acteoside definitely induced DNA fragmen-

tation with multiples of 180—200 base pair as actinomycin D, indicating that the cell death induced by acteoside is apoptosis. Furthermore, flow cytometric analysis revealed that the DNA content of the G_0/G_1, S and G_2/M phases decreased with incubation time and that, in contrast, DNA with a low fluorescence intensity, which represents degraded smaller DNA, significantly appeared 4 h after acteoside treatment. These results suggest that DNA cleavage occurred at an early time of cell death, in a distinct manner of apoptosis throughout the cell cycle (Fig. 4).

DISCUSSION

In this study we have found that acteoside elicited apoptosis in HL-60 cells, evidenced by the results of flow cytometric analysis and agarose gel electrophoresis. Apoptosis is an active form of cell death and is induced by the activation of cysteine proteases called caspases,[13] ceramide formation by sphingomyelinase,[14] activation of MAP kinase cascade,[15,16] and the generation of reactive oxygen species (ROS).[17,18] However, the precise mechanism by which apoptosis is induced and the existence of a common denominator is still obscure. Recent studies have shown that when the intensity of the insult is very strong and/or when ATP generation is deficient, cells fail to execute the ordered changes observed in apoptosis, resulting in the occurrence of cell lysis before the processes leading to nuclear condensation and fragmentation are complete.[12] In fact, many signal substances such as protein synthesis inhibitor,[19] glutamate,[20] nitric oxide,[21] oxidative stress[22] can induce both apoptosis and necrosis. In the case of acteoside, even more than 500 μg/ml of acteoside couldn't induce necrosis (data not shown), indicating that acteoside produces a moderate signal to elicit apoptosis but not necrosis.

Plant phenols are well known to show antioxidative activity which is usually believed to protect cells from oxidative stress. Many agents with antioxidative activity have been shown to be effective in suppressing cancer expansion in a

Acteoside (verbascoside)
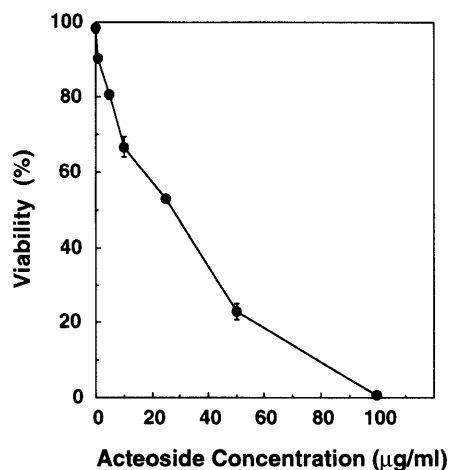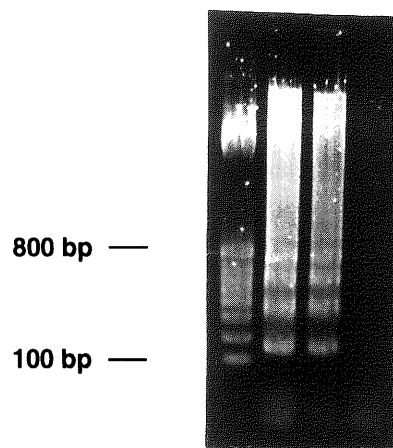
Fig. 1. Structure of Acteoside

Fig. 2. Cytotoxic Activity of Acteoside against HL-60 Cells

HL-60 cells at a concenration of 6×10^4 cells/ml were incubated with acteoside at various concentrations for 48 h. The viable cell number was determined by trypan blue dye exclusion method. Values represent means±S.E. of 4 wells.

STD ACT AD C

800 bp —

100 bp —

Fig. 3. Agarose Gel Electrophoresis of DNA Extracted from HL-60 Cells Exposed to Acteoside

HL-60 cells (6×10^6 cells) were treated with 50 μg/ml of acteoside for 6 h. DNA was isolated and subjected to electrophoresis on 1.5% agarose gel as described in Materials and Methods. STD, molecular weight marker; ACT, acteoside; AD, actinomycin D; C, control.
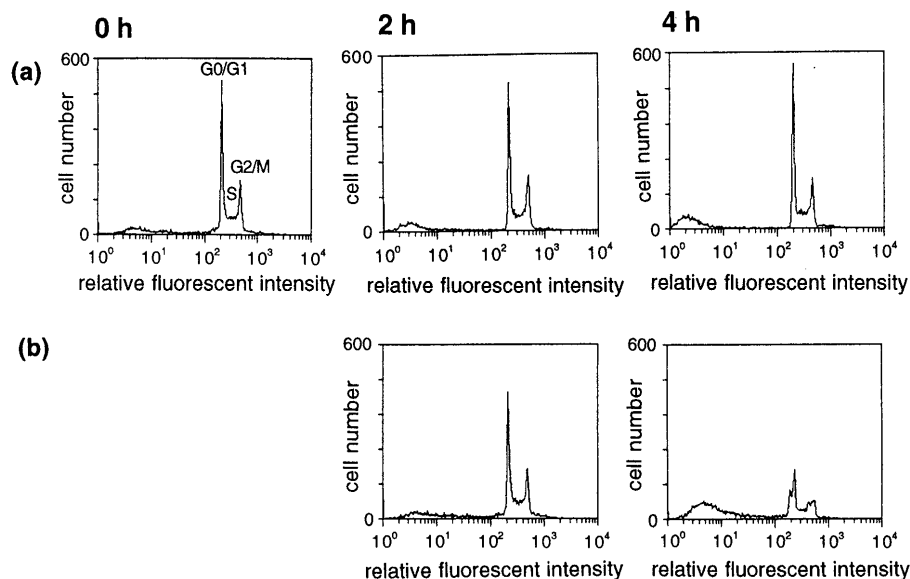
0 h **2 h** **4 h**

(a)

(b)

Fig. 4. Flow Cytometric Analysis of Cell Cycle Phase Distribution

HL-60 cells were treated with 50 μg/ml of acteoside for the indicated time. DNA content in each cell cycle phase was determined by FACScan as described in Materials and Methods. (a) control; (b) acteoside.

rodent hepatocarcinogenesis model. Antioxidants are considered to act as anticarcinogens or antimutagens by interacting with the carcinogen itself or with a carcinogen-produced reactive oxygen species. On the other hand, polyphenols are known to act as a pro-oxidant in the presence of metal ions and to cause damages to cells. Actually, caffeic acid, a component of acteoside, causes metal-dependent damage to cellular and isolated DNA through H_2O_2 formation.[23] Compounds which contain a catechol-like aromatic ring with two hydroxyl groups are known to autooxidize to produce hydrogen peroxide, which is capable of inducing DNA damage.[24] Both caffeic acid and chlorogenic acid are genotoxic, causing chromosome aberration in mammalian cells, gene conversion in yeast and in the presence of manganese, and reverse mutation in bacteria[25]; however such activities were not reported on acteoside. There is a possibility, though, that acteoside elicits a pro-oxidant action, resulting in the induction of apoptosis. Our previous work showed evidence that acteoside doesn't cause damage to primary cultured rat hepatocytes, suggesting that acteoside shows selectivity in its cytotoxic activity between normal cells and cancer cells. Furthermore, acteoside showed a biphasic effect on cancer cells, that is, cytostatic and/or cytotoxic activity, depending on the types of cells, indicating that all cancer cells are not always sensitive to acteoside. These observations present the possibility that the activity of acteoside depends mainly on the redox state in cells or that its activity is derived from a specific characteristic depending on its structure. However, the detailed mechanism underlying acteoside-induced apoptosis and the mode of cell death other than to HL-60 cells are currently under investigation.

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