Phloretin-induced Apoptosis in B16 Melanoma 4A5 Cells and HL60 Human Leukemia Cells

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Received October 22, 1998; Accepted December 8, 1998

The dihydrochalcone phloretin induced apoptosis in B16 mouse melanoma 4A5 cells and HL60 human leukemia cells. Phloretin was suggested to induce apoptosis in B16 cells mainly through the inhibition of glucose transmembrane transport. The phloretin-induced apoptosis in B16 cells was inhibited by actinomycin D, Ac-YVAD-CHO caspase-1-like inhibitor, and Ac-DEVD-CHO caspase-3-like inhibitor. During the induction of apoptosis by phloretin, the expression of Bax protein in B16 cells increased and the levels of p53, Bcl-2, and Bcl-X\textsubscript{L} proteins did not change. Our results suggested that phloretin induced apoptosis through the promotion of Bax protein expression and caspases activation. On the other hand, phloretin may induce apoptosis in HL60 cells through the inhibition of protein kinase C activity because phloretin inhibited protein kinase C activity in HL60 cells more than that in B16 cells. The phloretin-induced apoptosis in HL60 cells was not inhibited by actinomycin D and the caspase-1-like inhibitor, but slightly inhibited by the caspase-3-like inhibitor. Phloretin reduced the level of caspase 3 protein in HL60 cells, but not the level of the Bcl-2 protein. Phloretin did not increase the level of Bax protein. Phloretin was suggested to induce apoptosis in HL60 cells through the inhibition of protein kinase C activity, followed by the pathway, which is different from that in B16 cells.

Key words: phloretin; apoptosis; melanoma cells; leukemia cells; glucose transport

The naturally occurring dihydrochalcone, phloretin, inhibited tumor cell growth in vitro and in vivo. Phloretin inhibited the growth of Mol-M 4 human leukemic cells in vitro,12 and Fisher bladder carcinoma and rat mammary adenocarcinoma cells in vivo.23 However, the mechanism of tumor cell death induced by phloretin is not known. Recently we found that phloretin induced apoptosis in B16 mouse melanoma 4A5 cells.39 This finding suggests that phloretin inhibits tumor cell growth by induction of apoptosis in the cells.

Phloretin competitively inhibited glucose transport into erythrocytes, intestine, and brain.4-6) In our previous study, the addition of excess glucose into the medium inhibited the phloretin-induced apoptosis in B16 melanoma cells. Furthermore, cytochalasin B, which inhibits glucose transport and polymerization of actin, and diethylstilbestrol, which inhibits glucose transport, induced apoptosis in the B16 cells, while 2-deoxy-D-glucose, which inhibits glucose metabolism but not glucose transport, did not induce apoptosis. From these results, phloretin was suggested to induce apoptosis in B16 cells mainly through the inhibition of glucose transmembrane transport.31

The bioflavonoid genistein induced apoptosis in HL60 human leukemia cells, HT29 human colon cancer cells, and some other cells.5-10) Genistein was suggested to induce apoptosis through inhibition of protein tyrosine kinase and/or topoisomerase II.5,9) Another plant flavonoid, queretin, induced apoptosis in cancer cells, such as HT29 human colon cancer cells, HL60, and K562 human leukemia cells.10-12) The queretin-induced apoptosis has been supposed to be related to the inhibition of heat shock protein or phosphoinositides production.11,12) The structure of phloretin is closely related to these flavonoids, but phloretin inhibits protein kinase C (PKC) activity rather than protein tyrosine kinase activity.23 Phloretin is not as potent an inhibitor of protein tyrosine kinase and topoisomerase II as genistein.13) Moreover, the inhibition of glucose transmembrane transport, which is suggested to cause the apoptosis in B16 cells, is an effect unique to phloretin and not common among flavonoids. Hence phloretin must induce apoptosis in cancer cells through a unique pathway that differs from other flavonoid-induced pathways.

The PKC inhibitors, such as H7, staurosporine, and 7-hydroxystaurosporine, have induced apoptosis in human leukemia cells, colon carcinoma cells, and some other cancer cells.14-19 Therefore, phloretin may induce apoptosis in human leukemia and other cell lines through the inhibition of glucose transport or PKC.

To clarified the characteristics of phloretin-induced apoptosis in cancer cells, we examined the apoptosis inducing effect of phloretin in HL60 human leukemia cells, and some other cell lines. We also examined the effects of phloretin on the expression of p53, Bax, Bcl-2, and Bcl-X\textsubscript{L} proteins, and the activation of caspases. Our results suggested that phloretin induced apoptosis in B16 cells through promotion of the Bax expression and the activation of caspases. Phloretin induced apoptosis in HL60 cells through a pathway that differs from the phloretin-induced apoptosis pathway in B16 cells.
Materials and Methods

Chemicals. Phloretin (2',4',6'-trihydroxy-3-(p-hydroxyphenyl)propio phenone) was purchased from Funakoshi Chemicals Co. Ltd. H7 (1-(5-isouinolinylsulfonyl)-2-methylpipеразине) and actinomycin D were purchased from Sigma Chemical Co. Ltd. (St. Louis, USA). The caspase-1-like inhibitor, acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) was purchased from the Peptide Institute, Inc. (Osaka, Japan). The caspase-3-like inhibitor, acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). N-acetyl-L-cysteine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N-acetyl-L-cysteine was dissolved in PBS, but other chemicals were dissolved in dimethyl sulfoxide. Then these chemicals were added to a culture medium containing the cells.

Cells and cell culture. B16 mouse melanoma 4A5 cells (RCB557) was provided by the Riken Cell Bank (RCB, Ibaraki, Japan). Human promyelocytic leukemia HL60 cells (JCRB0085) were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). Human lung adenocarcinoma A-549 cells (JCRB0076) were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Chinese hamster lung CHL/1U cells were provided by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). B16 melanoma 4A5 cells and A-549 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan). HL60 cells were cultured in RPMI1640 (Nissui, Japan) and CHL/1U cells were grown in Eagle's minimum essential medium (MEM; Nissui, Japan). Cells were cultured at 37°C in the presence of 5% CO2, and the media were supplemented with 10% heat-inactivated fetal calf serum (FCS; Bioserum, Australia). Viable cells were counted with a hemocytometer by trypan blue exclusion. The cells treated with or without phloretin were fixed with 1% glutaraldehyde in phosphate buffered saline (PBS; Nissui, Japan) and the nuclei were stained with 1 μm Bisbenzimide (Hoechst) 33258 (Wako Pure Chemical Industries, Japan) in PBS.

DNA extraction and agarose gel electrophoresis. Cells (1 × 10⁶ cells) were lysed in 1 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 0.1 M EDTA and 0.5% SDS) and incubated with 0.05 mg/ml RNase at 37°C for 30 min, and then incubated with 0.1 mg/ml proteinase K at 37°C for 30 min. DNA was extracted in phenol, precipitated in ethanol, and resuspended in buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The samples were electrophoresed in Tris-borate buffer (pH 8.0) on a 2% agarose gel and the DNA was stained with ethidium bromide.

Flowcytometric analysis. Flowcytometric analysis was done to count hypodiploid cells. The cells were stained with propidium iodide using a Cycle Test Plus DNA Reagent Kit (Becton Dickinson, USA), then analyzed by FACScan (Becton Dickinson, USA) with Cell Fit software. Cells with DNA content less than G1 in the cell cycle distribution were counted as hypodiploid cells.

Western blot analysis. Cells were resuspended in PBS. The suspension (1 × 10⁶ cells/ml) was lysed in the same volume of 2 × loading buffer (20 mM Tris-HCl, pH 6.8, 2% SDS, 2% mercaptoethanol, and 20% glycerol), heated to 65°C for 10 min and sonicated. About 200 μl of the suspension was lysed in the same volume of buffer (20 mM Tris-HCl, pH 6.8, 2% SDS) to measure the protein concentration. After heat treatment (65°C, 10 min) and sonication, the protein content of each lysate was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cells extracts, containing equal amounts of protein, were electrophoresed in a 10% or 12.5% SDS-polyacrylamide gel. Then proteins were electrophoretically transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, England) and detected using antibodies and ECL detection system (Amersham).

Antibodies. Mouse anti-p53 monoclonal antibody (3a 240) was purchased from NeoMarkers (Fremont, CA, USA). Rabbit anti-Bax polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Mouse anti-Bcl-2 monoclonal antibody and mouse anti-mouse Bcl-x were purchased from Transduction Laboratories (Lexington, KY, USA). Rabbit anti-Bcl-x polyclonal antibody and mouse anti-human CPP32 (Ab-1) monoclonal antibody were obtained from Oncogene Research Products (Cambridge, MA, USA). Mouse anti-Bcl-2 monoclonal antibody and goat anti-rabbit IgG peroxidase were from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Sheep anti-mouse IgG1 peroxidase and sheep anti-mouse IgG2b peroxidase were from The Binding Site Limited (Birmingham, England).

PKC activity in cells. Cells were suspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.3% (w/v) 2-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 0.05 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 10 mM benzamidine). The suspension (2 × 10⁶ cells/ml) was lysed on ice by sonication and centrifuged at 10,000 × g for 1 h at 4°C. The supernatant (22.5 μl) was incubated with a different concentration of phloretin (2.5 μl), 25 μl of the reaction buffer (50 mM Tris-HCl, pH 7.5, 12 mM calcium acetate, 30 mM dithiothreitol, 0.3 mg/ml α-phosphatidyl-L-serine, 24 μg/ml phosphol 12-mycristate 13-acetate, 900 μM substrate peptide) and 5 μl of 1.2 mM [β-32P] ATP (0.4 μ Ci) for 15 min at 37°C. The reaction was stopped by the addition of 300 mM orthophosphoric acid. [32P] Phosphorylated peptides in the reaction mixture were bound to peptide binding paper and counted using 10 ml of Aqueous Counting Scintillant (ACS II, Amersham) and a Tri Carb 1900TP liquid scintillation analyzer (Packard, Meriden, CT). The chemicals and reagents were purchased from Amersham (England) as a PKC assay system.
Results

In our previous study we found that phloretin induced apoptosis in B16 mouse melanoma cells. Phloretin induced hypodiploid cells and internucleosomal DNA fragmentation in B16 cells. To clarify the apoptosis-inducing effect of phloretin in some human and animal cells, we measured the induction of hypodiploid cells by phloretin in HL60 human leukemia cells, A-549 human lung adenocarcinoma cells, and CHL/1U Chinese hamster lung cells. Treatment with 0.2 mM phloretin for 12 h induced 40% hypodiploid cells in HL60 cells (Table 1). However, when the cells were treated with 0.3 mM phloretin for 24 h, the ratios of hypodiploid cells in A549, and CHL/1U cells were only 12, and 9%, respectively. These results suggested that phloretin induced apoptosis in B16 and HL60 cells, but did not induce apoptosis in A549, and CHL/1U cells significantly. Phloretin inhibited the growth of HL60 cells at the concentrations of 0.1 and 0.2 mM during 6–48 h (Fig. 1). After 24 h of incubation of the cells with 0.2 mM phloretin, condensation and fragmentation of nuclei were observed in HL60 and B16 cells (Fig. 2). DNA fragmentations typical for apoptosis also bear out the induction of apoptosis in these cells by phloretin (Fig. 1). Thus phloretin was shown to induce apoptosis in both B16 and HL60 cells.

Phloretin has been suggested to induce apoptosis in B16 cells mainly through the inhibition of glucose transmembrane transport because the addition of extracellular glucose inhibited the induction of apoptosis. On the contrary, the addition of extracellular glucose did not affect phloretin-induced apoptosis in HL60 cells (data not shown). Thus, the inhibition of glucose transmembrane transport was suggested not to be a major trigger of phloretin-induced apoptosis in HL60 cells. Phloretin may induce apoptosis through inhibition of PKC activity in HL60 cells because some of the PKC inhibitors induce apoptosis in HL60 cells. The PKC activator 12-o-tetradecanoyl-phorbol-13-acetate (TPA) did not inhibit the phloretin-induced apoptosis in HL60 cells (data not shown). However phloretin inhibited the PKC activity in HL60 cells more than the activity in B16 cells (Fig. 3). Furthermore the PKC inhibitor H7 is readily induces apoptosis in HL60 cells. When HL60 cells were incubated with 0.2 mM H7 for 12 h, 50–60% hypodiploid cells were induced. Phloretin may induced apoptosis in the HL60 cells not through the inhibition of glucose transport but by inhibiting PKC activity.

The B16 cells were treated for 2 h with the RNA synthesis inhibitor actinomycin D, the caspase-1-like inhibitor Ac-YVAD-CHO, or the caspase-3-like inhibitor Ac-DEVD-CHO, then treated with 0.2 mM phloretin for 22 h. These inhibitors suppressed the induction of apoptotic cells from 48% to 10–25% of the total numbers of cells (Fig. 4A). Then we measured the expression of proteins related to apoptosis induction in phloretin-treated B16 cells. When B16 cells were treated with 0.2 mM phloretin, the amount of Bax protein was clearly increased time dependently during 0–18 h (Fig. 4B).

### Table 1. Phloretin Induced Hypodiploid Cells in Human and Animal Cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Animal and Cell type</th>
<th>% Hypodiploid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>HL60</td>
<td>Human leukemia</td>
<td>10.9±0.4</td>
</tr>
<tr>
<td>A-549</td>
<td>Human lung adenocarcinoma</td>
<td>4.7±0.5</td>
</tr>
<tr>
<td>CHL/1U</td>
<td>Chinese hamster lung</td>
<td>2.5±0.1</td>
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HL60 cells were treated with or without 0.2 mM phloretin for 12 h. A-549, and CHL/1U cells were treated with or without 0.3 mM phloretin for 24 h. Numbers of hypodiploid cells were calculated by DNA fluorescent histograms. Results are expressed as means±SD of triplicate samples.
the other hand, the expressions of Bcl-2 and Bcl-x were not significantly affected by phloretin. The p53 protein was reported to be expressed in B16 cells. However, no increase of the Bax expression level was detected.

Actinomycin D and caspase-1-like inhibitor did not inhibit the phloretin-induced apoptosis in HL60 cells (Fig. 5A). The antioxidant N-acetylcysteine did not suppress the phloretin-induced apoptosis, either. The caspase-3-like inhibitor inhibited only about 13–15% of the apoptotic cell-induction at the concentration of 0.1 or 0.2 mM (Fig. 5A). This result suggested that caspase-3-like proteases are activated during the induction of apoptosis by phloretin though the effect of caspase-3-like inhibitor in HL60 cells was weaker than that in B16 cells. In order to confirm the participation of caspase 3 in phloretin-induced apoptosis in HL60 cells, the expression of caspase 3 was measured during the induction of apopto-

Fig. 2. Nuclear Morphology of HL60 Cells and B16 Melanoma 4A5 Cells.

The cells were treated with or without 0.2 mM phloretin for 24 h and stained with Hoechst 33258. A: HL60 cells B: HL60 cells treated with 0.2 mM phloretin C: B16 cells D: B16 cells treated with 0.2 mM phloretin.

Fig. 3. Effects of Phloretin on the Protein Kinase C Activity in Crude Cell Preparations of HL60 Cells and B16 Melanoma 4A5 Cells.

Crude cell extract (1.0 × 10⁶ cells/ml) was prepared and exposed to phloretin for 15 min at 37°C. Protein kinase C activity was measured using a protein kinase C enzyme assay system (Amersham).

Fig. 4. (A) Effects of Inhibitor on Phloretin-induced Hypodiploid Cells in B16 Melanoma 4A5 Cells.

Cells were treated with each inhibitor for 2 h and then treated with 0.2 mM phloretin for 24 h.

(B) Effects of Phloretin on Expression of Bax, Bcl-2 and Bcl-XL Protein in B16 Melanoma 4A5 Cells.

Protein Levels in the Cells Treated with 0.2 mM Phloretin for 0–18 h were Examined by Western Blot Analysis.
Fig. 5. (A) Effects of Inhibitor on Phloretin-induced Hypodiploid Cells in HL60 Cells.
Cells were treated with each inhibitor for 2 h and then treated with 0.25 mM phloretin for 24 h.
(B) Effects of Phloretin on Expression of Caspase 3, Bcl-2 and Bcl-XL Protein in HL60 Cells.
Protein levels in the cells treated with 0.25 mM phloretin for 0–18 h were examined by Western blot analysis.

Phloretin-induced Apoptosis in B16 Cells and HL60 Cells

Phloretin inhibited apoptosis in B16 mouse melanoma cells. HL60 human leukemia cells, in which apoptosis is readily induced by various components, were able to be induced by phloretin, while A-549 human lung cells and CHL1/IU Chinese hamster lung cells were not. Phloretin was shown to inhibit the D-glucose transport activity of GLUT1 glucose transporter and competitively inhibited glucose transmembrane transport into erythrocytes. Phloretin inhibited the uptake of glucose by tumor cells in vitro and in vivo and inhibited the growth of the cells. Among these 4 cell lines, only B16 cells were suggested to have apoptosis induced through glucose transmembrane transport. B16 cells may be very sensitive to inhibition of glucose transmembrane transport. On the other hand, phloretin was suggested to induce apoptosis in HL60 cells not through the inhibition of glucose transport because extracellular glucose did not inhibit the induction of apoptosis.

The antioxidant N-acetylcysteine inhibited the apoptosis that was induced by tumor necrosis factor-α, nitric oxide or lactoferrin, in human leukemia cells.

Although flavonoids are easily oxidized and have antioxidant properties, N-acetylcysteine did not inhibit phloretin-induced apoptosis in HL60 cells. Phloretin was suggested to induce apoptosis in HL60 cells, but not through oxidative stress.

Many reports have shown that inhibition of PKC is a common pathway of induction of apoptosis in HL60 cells. Phloretin strongly inhibited the PKC activity in HL60 cells, in vitro. Although we must further confirm the suppression of the PKC activity by phloretin during the apoptosis in HL60 cells, phloretin is likely to induce apoptosis in HL60 cells by inhibition of PKC activity.

UV irradiation of B16 cells increased the expression of the p53 tumor-suppressor-gene product and induced the apoptosis with DNA fragmentation. However phloretin did not increase the level of the p53 protein in B16 cells. p53 protein remained at undetectable levels during the induction of apoptosis by phloretin. Bax is known to accelerate apoptosis and form homo- and heterodimers with Bcl-2 or Bcl-XL. The Bax protein level was often increased following the increase of p53 expression because p53 is a transcriptional activator of the bax gene, while overexpression of the Bax protein accelerated apoptosis in a human ovarian cancer cell line through a p53-independent pathway. However, phloretin increased the level of Bax protein in B16 cells without increasing the p53 protein level. Phloretin did not affect the levels of Bcl-2, which suppresses apoptosis, and Bcl-XL, which inhibits apoptosis, independently of Bcl-2 expression. Bax has been reported to induce apoptosis through a caspase-dependent or independent pathways. The results of inhibitory experiments using the substrates of caspase-1-like proteases and caspase-3-like proteases suggest that caspase-1-like and caspase-3-like proteases were activated during the induction of apoptosis in B16 cells. Although it was not demonstrated that the amount of Bax protein induced by phloretin is enough to accelerate apoptosis in B16 cells, phloretin was suggested to induce apoptosis through induction of Bax expression and followed by the activation of caspases. The suppression of phloretin-induced apoptosis by the RNA synthesis inhibitor actinomycin D also suggests that the synthesis of Bax protein is required for phloretin-induced apoptosis. The phloretin-induced apoptosis in B16 cells seems to be independent of the regulation of Bcl-2, Bcl-XL and p53 expressions.

Apoptosis in HL60 cells is known to be independent of p53 as HL60 cells are p53 null. Phloretin did not increase the level of Bax protein in HL60 cells and therefore phloretin-induced apoptosis in HL60 cells was
shown to be independent of Bax. Down-regulation of Bel-2 was suggested to cause the apoptosis in HL60 cells, but the PKC inhibitors H7 and staurosporine induced apoptosis in HL60 cells through Bel-2 independent pathways. Phloretin-induced apoptosis in HL60 cells were independent from Bel-2 similar to the protein kinase inhibitors, staurosporine and H7. On the other hand, we observed the reduction of the Bel-XL protein level after 12 or 18 h of treatment. The down-regulation of Bel-XL is not likely to cause apoptosis in HL60 cells as the reduction can be observed only in the very late stage of phloretin-induced apoptosis. Many types of apoptotic pathways involve the activation of caspases. The protein kinase inhibitor 7-hydroxysteruasporine-induced apoptosis in HL60 cells was associated with caspases activation. In our results, the caspase-1-like inhibitor did not inhibit the phloretin-induced apoptosis in HL60 cells and the caspase-3-like inhibitor slightly inhibited the apoptosis. However, caspase 3 was suggested to be proteolytically activated in HL60 cells, because the level of the caspase 3 protein was decreased during the induction of apoptosis by phloretin. It is suggested that the caspase 3 activation is involved in the phloretin-induced apoptosis in HL60 cells.

Phloretin thus induced apoptosis in B16 melanoma cells and in HL60 cells. Phloretin was suggested to induce apoptosis in B16 cells through the inhibition of glucose transmembrane transport and induction of the Bax protein expression and the caspase activations. On the other hand, phloretin was suggested to induced apoptosis in HL60 cells through this pathway, which is different from that in B16 cells. Phloretin must inhibit the growth of cancer cells in vitro and in vivo by the induction of apoptosis through the inhibition of glucose transport or PKC, followed by the various pathways.

References


