Flavonoids Inhibit Cell Growth and Induce Apoptosis in B16 Melanoma 4A5 Cells

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We investigated the growth inhibitory activity of several flavonoids, including apigenin, luteolin, kaempferol, quercetin, butein, isoliquiritigenin, naringenin, genistein, and daizein against B16 mouse melanoma 4A5 cells. Isoliquiritigenin and butein, belonging to the chalcone group, markedly suppressed the growth of B16 melanoma cells and induced cell death. The other flavonoids tested showed little growth inhibitory activity and scarcely caused cell death. In cells treated with isoliquiritigenin or butein, condensation of nuclei and fragmentation of nuclear DNA, which are typical phenomena of apoptosis, were observed by Hoechst 333258 staining and by agarose gel electrophoresis of DNA. Flow cytometric analysis showed that isoliquiritigenin and butein increased the proportion of hypodiploid cells in the population of B16 melanoma cells. These results demonstrate that isoliquiritigenin and butein inhibit cell proliferation and induce apoptosis in B16 melanoma cells. Extracellular glucose decreased the proportion of hypodiploid cells that appeared as a result of isoliquiritigenin treatment. p53 was not detected in cells treated with either of these chalcones, however, protein of the Bcl-2 family were detected. The level of expression of Bax in cells treated with either of these chalcones was markedly elevated and the level of Bcl-X decreased slightly.

Isoliquiritigenin did not affect Bcl-2 expression, but butein down-regulated Bcl-2 expression. From these results, it seems that the pathway by which the chalcones induce apoptosis may be independent of p53 and dependent on proteins of the Bcl family. It was supposed that isoliquiritigenin induces apoptosis in B16 cells by a mechanism involving inhibition of glucose transmembrane transport and promotion of Bax expression. On the other hand, it was suggested that butein induces apoptosis via down-regulation of Bcl-2 expression and promotion of Bax expression. This mechanism differs from the isoliquiritigenin induction pathway.

Key words: flavonoid; antiproliferation; apoptosis; melanoma cells; Bcl-2 family

Dietary flavonoids, commonly present in edible plants, are known to have beneficial effects, for example, antioxidative effects, tumor cell growth inhibitory activity, and activity in induction of apoptosis in cancer cell lines. Therefore dietary flavonoids have attracted attention as chemopreventive agents.

It is reported that some flavonoids have antiproliferative activity and induce apoptosis in cancer cell lines. Quercetin, a flavonoid commonly present in many vegetables, has been shown to induce apoptosis in various tumor cell lines including HL60 or K562 human leukemia cells and HT29 human colon cancer cells. Genistein, which is known to resemble estrogen in structure, has been found to inhibit the proliferation of estrogen receptor-positive MCF-7 human breast cancer cells. Genistein was also found to induce apoptosis in HL60 human leukemia cells and HT29 human colon cancer cells.

Genistein and other bioactive constituents of edible plants are known to influence melanocytes and melanoma cells. The soybean isoflavone genistein has been shown to significantly inhibit the growth of human melanoma cell lines. Genistein causes the melanoma cell cycle to be arrested at the G2/M point, but it does not induce apoptosis in these cells. Watabayaishi et al. have reported that a ginseng metabolite, M1, inhibited the growth of B16 BL6 mouse melanoma cells and induced apoptosis in these cells. The metabolite M1 was found to up-regulate a cyclin-dependent kinase inhibitor and to down-regulate the apoptosis-related factors c-Myc and cyclin D1 through transcriptional regulation. Another component, saikosaponin b2, has been found to induce apoptosis by down-regulation of PKC activity in B16 melanoma cells.

In our previous report, we demonstrated that the dihydrochalcone phloretin, a main apple flavonoid,
induced apoptosis in B16 melanoma cells through the inhibition of glucose transmembrane transport.\textsuperscript{13} Our findings supported the view that phloretin induces apoptosis via promotion of Bax protein expression and activation of caspases.\textsuperscript{10}

In this study, we investigated the antiproliferative activity of other dietary flavonoids against B16 mouse melanoma cells. We found that isoliquiritigenin and butein markedly inhibited cell growth and induced apoptosis in these cells. To clarify the mechanism of induction of apoptosis, we investigated the expression of apoptosis-promoting and apoptosis-inhibiting proteins in B16 cells treated with isoliquiritigenin or butein.

Materials and Methods

Chemicals. Isoliquiritigenin was purchased from Sigma Chemical Co. Ltd. (St. Louis, USA). Quercetin and naringenin were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Other flavonoids were purchased from Funakoshi Chemicals Co. Ltd. (Tokyo, Japan). These flavonoids were dissolved in dimethylsulfoxide and then added to the culture medium. Mouse anti-p53 monoclonal antibody (Pab 240, type IgG1) was purchased from Neomarkers (Fremont, CA, USA). Mouse anti-Bcl-2 (type IgG1) and anti-Bcl-X (type IgG2b) monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY, USA). Rabbit anti-Bax polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated antibodies, goat anti-rabbit IgG1, sheep anti-mouse IgG1, and IgG2b were purchased from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan).

Cell Culture. B16 mouse melanoma 4A5 cells (RCB557, B16 cells) were provided by the RIKEN Cell Bank (RCB, Ibaraki, Japan). The B16 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan), with 10% heat-inactivated fetal calf serum (FCS; JRH Biosciences, KS, USA). The B16 cells were cultured in a CO\textsubscript{2} incubator (37°C, 5\%CO\textsubscript{2}). Viable cells, as identified by trypan blue exclusion, were counted with a hemocytometer. Morphological changes in cells treated with flavonoids were observed by microscopy. The cells incubated with isoliquiritigenin or butein were fixed and stained by the same procedure as described in our previous report.\textsuperscript{10} Then the condensation of nuclei in the cells was observed.

DNA extraction and agarose gel electrophoresis. One × 10\textsuperscript{6} cells were lysed in 20 \textmu l of lysis buffer (50 mM Tris-HCl (pH 8.0), 0.01 M EDTA·Na and 0.5\% SDS). Five \textmu l of RNase solution (10 mg of RNase/ml lysis buffer) was added to the cell lysate and the mixture was incubated at 50°C for 30 min. Thereafter, 5 \textmu l of proteinase K solution (10 mg of proteinase K/ml lysis buffer) and mixture was incubated at 50°C for 60 min. The DNA solution was fractionated by electrophoresis on a 2\% agarose gel in Tris-borate buffer (pH 8.0). After electrophoresis, the agarose gel was incubated with ethidium bromide to make the DNA visible.

Flow cytometric analysis. Hypodiploid cells have less DNA than that of diploid cells at G1 phase because of apoptosis-induced DNA fragmentation. After incubation with the flavonoids for 24 h, the proportion of hypodiploid cells was measured by a flow cytometer. Propidium iodide-stained cells were prepared using a Cycle Test Plus DNA Reagent Kit (Becton Dickinson, CA, USA). Flow cytometry was done with FACScan (Becton Dickinson) and the proportion of hypodiploid cells was analyzed by using Cell Fit software (Becton Dickinson).

Western blot analysis. Cells incubated with either of the chalcones were collected and suspended in PBS to be 1 × 10\textsuperscript{7} cells/ml and the cell suspension was divided into two tubes. The same volume of 2 × loading buffer (20 mM Tris-HCl, pH 6.8, 2\% SDS, 2\% 2-mercaptoethanol, and 20\% glycerol) was added to the cell suspension in one tube to induce cell lysis. The lystate was heated at 65°C for 10 min and sonicated. The same volume of buffer (20 mM Tris-HCl, pH 6.8, 2\% SDS) was added to the other tube to measure the protein concentration. After heat treatment and sonication under the same conditions, the protein content of each lystate was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). After the protein assay, the proteins in aliquots of the cell extracts were size-fractionated by electrophoresis on a 10\% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). The expression of apoptosis-promoting and apoptosis-inhibiting proteins was detected by means of antibodies and the ECL detection system (Amersham).

Results

We investigated the growth inhibitory effect of flavonoids on B16 melanoma cells. The classes and structures of the flavonoids investigated in this study are shown in Fig.1. Figure 2 shows the growth inhibitory effect of the flavonoids at 100 \mu M and 200 \mu M. Upon addition of naringenin or daizein to the medium, the number of viable cells was less than that in the case of the untreated control after 12 h. However, the total number of viable cells finally reached a level near that of the control after 24 h of incubation. Upon addition of quercetin, genistein,
apigenin, kaempherol, or luteolin, the number of viable cells did not increase to the control level as measured after 24 h of incubation. These flavonoids inhibited the proliferation of the B16 cells, but the number of viable cells did not decrease. These flavonoids seemed to have little effect in terms of causing cell death.

When B16 cells were treated with 200 μM phloretin, the number of viable cells decreased (Fig. 2). Isoliquiritigenin and butein, belonging to chalcone group as does phloretin, inhibited cell growth and caused a marked decrease in the number of viable B16 cells at a concentration of 200 μM (Fig. 3). We tried to clarify whether cell death was caused by induction of apoptosis or not. DNA fragmentation and chromatin condensation are known as classical signs of apoptosis. Therefore, the DNA pattern and changes in nuclear morphology were examined. The B16 cells were incubated with isoliquiritigenin or butein for 24 h. Then the DNA in the B16 cell lysate was fractionated by agarose gel electrophoresis and observed under UV after staining with ethidium bromide (Fig. 3). Ladder patterns, typical of apoptosis, were observed in the case of the agarose gel to which the B16 cell lysate had been applied. The B16 cells were fixed and stained with Hoechst 33258 (Fig. 4). In the case of the isoliquiritigenin or butein-treated B16 cells, chromatin condensation was evident under a fluorescence microscope. These results demonstrated that isoliquiritigenin and butein induced apoptosis in B16 cells.

We then examined the cell cycle of B16 cells undergoing apoptosis induced by isoliquiritigenin or butein as compared with that in the case of apoptosis induced by phloretin. The B16 cells after incubation for 24 h with the chalcones were stained with propidium iodide (PI), as described in Materials and Methods. The cells undergoing apoptosis were detected as hypodiploid cells which contained less DNA than that of diploid cells at G1 phase. The proportion of hypodiploid cells in the population was measured by flow cytometry (Fig. 5A). Upon treatment of the B16 cells with any of the three chalcones, isoliquiritigenin, butein, or phloretin, the proportion of hypodiploid cells increased in a concentration-dependent manner. At the concentration of 200 μM, the proportion of hypodiploid cells after isoliquiritigenin treatment was 84.3% and this was higher than that for butein (63.0%) or phloretin (57.0%). Isoliquiritigenin was the most potent of the three chalcones tested in this study (P < 0.01). The induction of apoptosis upon treatment of these cells with 150 μM phloretin has been shown to be inhibited by 100 mM extracellular glucose. Therefore, the effect of extracellular glucose on apoptosis induction by isoliquiritigenin and butein was examined. The proportion of hypodiploid cells in the case of 150 μM isoliquiritigenin treatment was diminished by 100 mM glucose (P < 0.01). On the other hand, 100 mM glucose scarcely affected the proportion of hypodiploid cells in the case of butein treatment.

The apoptosis pathway involves many factors. We examined the expression of some apoptosis-related proteins in B16 cells. Cells incubated with 200 μM isoliquiritigenin or butein were harvested during 18 h and the lysate was fractionated by SDS-PAGE. Protein expression was analyzed by western blotting. p53-dependent and p53-independent pathways are known to exist as major apoptosis pathways. However, the p53 protein was not detected in the B16 cells (data not shown).

Not only p53, but also proteins of the Bcl-2 family are important in regulation of apoptosis. Bax is known as a pro-apoptotic protein and it forms homo- or heterodimers with the anti-apoptotic proteins Bcl-2 and Bcl-X. Therefore, the levels of expression of Bcl-2 family proteins in the cells were measured. The level of expression of Bax in the B16 cells incubated with isoliquiritigenin or butein increased markedly in a time-dependent manner (Fig. 6). The level of expression of Bcl-2 in the isoliquiritigenin-treated
Fig. 2. Effects of Flavonoids on the Growth of B16 Melanoma 4A5 Cells. control, ●; 100 μM flavonoid, ▲; 200 μM flavonoid, ■.

cells remained constant, but the level in the butein-treated cells decreased with incubation time. The expression of Bcl-XL was weak in cells treated with either of these chalcones and nearly disappeared. The expression of apoptosis-related proteins differed, comparing the isoliquiritigenin- and butein-treated cells.

Discussion

Several flavonoids are known to inhibit cancer development in vivo and tumor cell growth in vitro.\textsuperscript{31} They may be important in cancer chemoprevention. Dietary flavonoids in edible plants can be further subdivided into several structural groups. The
Fig. 3. Effects of Isoliquiritigenin and Butein on the Growth of B16 Melanoma Cells and DNA Fragmentation Pattern Analysis by Agarose Gel Electrophoresis.

The incubation time was 24 h. control, ●; 100 μM flavonoid, ▲; 200 μM flavonoid, ■; IL, isoliquiritigenin; B, butein.

Fig. 4. Nuclear Morphology of B16 Melanoma 4A5 Cells Stained with Hoechst 33258.

The cells were incubated in the absence or presence of 200 μM isoliquiritigenin or 200 μM butein for 24 h.

Chemopreventive activity of flavonoids is dependent on their structural features. Kuo et al. have reported the activity of flavonoids in inhibiting the proliferation of Caco-2 cells and HT29 cells, which are colon cancer cell lines. They suggested that the activity was correlated with the structural features of...
these flavonoids. The double bond in the A-ring C2-3 and the hydroxyl group position were related to the potency. Also, Aguillo et al. have reported comparative effects of flavonoids on HT29 colon cancer.\textsuperscript{15} The cytotoxic potency against HT29 cells was found to be dependent on desaturation of the 2-3 bond and the position of attachment of the B-ring, as shown in Kuo’s report.

Isoliquiritigenin, 2',4',4-trihydroxychalcone, can be obtained from licorice, alfalfa, Pueraria lobata (kuzu), or Pismum sativum L. Isoliquiritigenin has potent anti-tumor-promoting action against skin tumors. The action by isoliquiritigenin is thought to act on cells through the inhibition of lipoxigenase activity.\textsuperscript{16} Butein is present in members of the Leguminoseae family, and it has an effect on proliferation of human colon adenocarcinoma cells,\textsuperscript{17} and HeLa cells.\textsuperscript{18} Butein has been shown to inhibit a specific protein tyrosine kinase, but not PKA or PKC, in HL-60 leukemia cells.\textsuperscript{19}

We found that isoliquiritigenin and butein inhibited B16 melanoma cell growth. Isoliquiritigenin or butein at 200 μM inhibited B16 cell proliferation and caused a marked decrease in the number of viable cells (Fig. 1). A decrease in the number of viable B16 cells was observed only in the case of the chalcones, isoliquiritigenin, butein, and phloretin among the flavonoids tested in this study. Quercetin and genistein induce apoptosis in some kinds of cell lines.\textsuperscript{3-10} But, in our case of B16 cells, quercetin, genistein, apigenin, kaempferol, and luteolin each inhibited cell proliferation but they seemed to induce little cell death. No particular structural features of flavonoids associated with the effects on B16 cells, like desaturation of a bond or hydroxyl group position, were observed among the flavonoids tested. The relationship between the growth inhibitory activity and the structural features of the flavonoids remains unclear.

In cells treated with isoliquiritigenin or butein, morphological changes were observed (data not shown). In the presence of 100 μM or 150 μM isoliquiritigenin or butein, the B16 cells were slimmer in shape than the control cells, as observed under the microscope. Most of the B16 cells were detached from the surface of the culture dish and were floating in the medium after treatment at 200 μM. We investigated whether B16 cell death induced by isoliquiritigenin or butein was attributable to apoptosis. A DNA ladder was observed after fractionation of the nuclear DNA by agarose gel electrophoresis under UV and chromatin condensation was observed under a fluorescence microscope (Fig. 2 and 3). On the basis of these hallmarks of apoptosis, we conclude that isoliquiritigenin and butein induce apoptosis in B16 cells. The cell death caused by the chalcones was suggested to be attributable to apoptosis.

The potency of the apoptosis induction activity was ranked as isoliquiritigenin > butein = phloretin on the basis of the results of flowcytometric analysis (Fig. 5A). The proportion of hypodiploid cells in the population of B16 cells treated with isoliquiritigenin was reduced in the presence of 100 mM extracellular glucose (P<0.01) (Fig. 5B). Isoliquiritigenin-induced apoptosis may involve inhibition of glucose transmembrane transport, as in the case of phloretin.\textsuperscript{10} On the other hand, butein-induced apoptosis was not affected by extracellular glucose. The apoptosis pathway in the case of butein-induced apoptosis does not involve inhibition of glucose transmembrane transport.

It is well-known that many factors are involved in the apoptosis pathway. To clarify the mechanisms of induction of apoptosis by isoliquiritigenin and butein, we investigated the expression of apoptosis-
Inhibiting and apoptosis-promoting proteins in B16 cells. A p53-dependent pathway and a p53-independent pathway are known to exist as major apoptosis pathways. X-rays and topoisomerase inhibitors induce apoptosis via the p53-dependent pathway. However, p53 was not detected in the isoliquiritigenin- or butein-treated cells (data not shown). Our findings suggest that induction of apoptosis by these chalcones occurs via a p53-independent pathway.

Proteins of the Bcl-2 family are known to regulate promotion and inhibition of apoptosis. Members of the Bcl-2 family are expressed in human melanocytes and melanoma cell lines. Skin cancers may develop through the loss of pro-apoptotic Bax protein. Selzer et al. reported that expression of Bcl-2 and its homologues is common in melanoma cell lines (in vivo) and normal human melanocytes (in vitro). They could not detect fragmentation of nuclear DNA in the case of human melanoma lymph node metastases. However, they demonstrated the pattern of expression of Bcl-2 and its homologues. Expression of Bcl-2, Bak and Bax is observed in most melanocytes and melanoma cell lines. The Bax protein is known to be an apoptosis induction factor because of the formation homo- and heterodimers with the apoptosis suppressing factors Bcl-2 and Bcl-X<sub>L</sub>. The loss of expression of the Bax protein in squamous cell carcinoma is considered to be an important step in skin tumor development. Expression of the Bax protein is thought to result in inhibition of melanoma development through induction of apoptosis.

In our previous report, expression of the Bax protein in phloretin-treated B16 cells was shown to increase time-dependently, playing an important role in apoptosis induction. In both isoliquiritigenin-treated and butein-treated cells, Bax protein expression showed strong time-dependency within 18 h as in the case of phloretin-treated cells. On the other hand, the level of expression of Bcl-X<sub>L</sub> decreased time-dependently in these cells. The level of expression of the anti-apoptotic protein Bcl-2 was stable in the isoliquiritigenin-treated cells though the level was lower in butein-treated cells time-dependently.

In summary, isoliquiritigenin and butein were found to inhibit the growth of B16 melanoma cells through induction of apoptosis. The mechanisms of induction of apoptosis by isoliquiritigenin and butein are thought to differ, and are p53 independent. The apoptosis-induction pathway in the case of isoliquiritigenin appears to have a possibility of inhibi-
tion of glucose transmembrane transport and involve promotion of Bax expression and down-regulation of Bcl-XL. Our findings suggested that butein down-regulates expression of Bcl-2 and Bcl-XL and up-regulates expression of Bax, and thereby induces apoptosis in B16 cells.

Acknowledgments

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References