Structure-activity Relationships of Flavonoids and the Induction of Granulocytic- or Monocytic-Differentiation in HL60 Human Myeloid Leukemia Cells

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The flavones apigenin and luteolin strongly inhibited the growth of HL60 cells and induced morphological differentiation into granulocytes. The flavonol quercetin inhibited the cell growth and induced a differentiation marker, i.e., NBT reducing ability. However quercetin-treated cells were not morphologically differentiated into granulocytes. The chalcone phloretin weakly induced NBT reducing ability and a marker of monocytic differentiation α-naphthyl butyrate esterase activity in the cells. Quercetin and phloretin appeared to induce the differentiation of HL60 cells into monocytes. The proportion of α-naphthyl butyrate esterase-positive cells induced by genistein was less than that of the NBT-positive cells. Some of the nuclei in genistein-treated HL60 cells morphologically changed. Genistein must have induced both granulocytic and monocytic differentiation of HL60 cells. The flavonoids galangin and kaempferol, which had fewer hydroxyl group(s) in the B-ring than quercetin, and the flavanone naringenin inhibited the growth but did not induce the differentiation of HL60 cells.

Key words: flavonoids; differentiation; HL60 human leukemia cells; apigenin; genistein

Flavonoids play a significant role in the physiological function of foods. Flavonoids contained in vegetables and fruits have antioxidant activity that protects tissues against oxygen free radicals and lipid peroxidation.1,2,3 The antioxidant activity of flavonoids is thought to help prevent heart disease and cancer.4,5 Thus, physiological functions of flavonoids are partly explained by their antioxidant activity. Flavonoids also directly inhibit the growth of cancer cells and induce the apoptosis and/or differentiation of cancer cells. Flavonoids inhibit the growth of human leukemia cells, colon adenocarcinoma cells, and breast cancer cells.5,7,8,9 Quercetin induces the apoptosis in human leukemia cells and colon cancer cells.5,10 Phloretin induces the apoptosis in B16 mouse melanoma cells by inhibiting glucose transmembrane transport.11 Genistein induces a G2/M cell-cycle arrest and apoptosis in leukemia cells,12,13,14 as well as the differentiation of HL60 and K562 human leukemia cells.15 Some flavonoids, such as apigenin, genistein, and naringenin, induce the differentiation of murine erythroleukemia cells into erythrocytes.16,17 Apigenin also induced the morphological differentiation and G2/M arrest in rat neuronal cells.10 Flavonoids thus may directly affect variant cancer cells and prevent cancer.

HL60 human leukemia cells can be induced to differentiate into granulocytes, monocytes, and macrophages by differentiation-inducing agents. Retinoic acid, which is used for clinical therapy of patients with acute promyelocytic leukemia, induces differentiation of HL60 cells into granulocytes.19,20 Vitamin D3 and sodium butyrate induce differentiation into monocytes, while phorbol esters induce differentiation of the cells into macrophages.18 The isoflavone genistein was reported to induce markers of the differentiation of HL60 cells, NBT reducing ability and α-naphthyl butyrate esterase activity.12 However, the effects of flavonoids on induction of HL60 cell differentiation are mostly unknown. To discover the structural requirements of flavonoids for granulocytic- and monocytic-differentiation induction we investigated the ability of flavonoids to induce differentiation in HL60 human myeloid leukemia cells.

Materials and Methods

Chemicals. Quercetin was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Other flavonoids (Apigenin, Luteolin, Galangin, Kaempferol, Phloretin, Naringenin, and Genistein) were purchased from Funakoshi Chemicals Co. Ltd. (Tokyo, Japan). Trypan blue was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Giemsa solution was obtained from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Nitroblue tetrazolium (NBT) was purchased from Wako Pure Chemical Industrial Co. (Osaka Japan).

Cells and Cell Culture. HL60 cells were provided by the Japanese Cancer Research Resource Bank (JCRB0085). The cells were maintained in RPMI1640 medium (Nipro Co. Ltd., Osaka, Japan) with 10% heat-inactivated fetal calf serum (FCS; Bioserum, Australia) at 37°C in a humidified atmosphere of 5% CO2 in air. The cells were plated in the medium at a density of 5 x 10⁴ cells/ml and grown for 4 or 5 days in the absence and presence of each flavonoid dissolved in dimethylsulfoxide (DMSO, Wako Pure Chemical Industrial Co., Osaka, Japan). Viability of cells was observed by trypan blue dye exclusion. The morphology of the cells was estimated by Giemsa staining.

Abbreviations: DMSO, Dimethylsulfoxide; NBT, Nitroblue tetrazolium; PKC, Protein kinase C; PTK, Protein tyrosine kinase.
Mesurement of Nitroblue Tetrazolium (NBT) Reducing ability and α-Naphthyl Butyrate Esterase Activity. NBT reducing ability in HL60 cells was evaluated by the method of Collins et al. The α-naphthyl butyrate esterase activity was measured by the method of Li et al. α-Naphthyl butyrate esterase positive cells were stained with 2-methyl-4-[(2-methylphenyl)-azobenzenediazonium (fast garnet GBC). NBT positive and α-naphthyl butyrate esterase positive cells in 200 HL60 cells were counted under a microscope.

Results

Figure 1 shows the structures of flavonoids that we evaluated as inducing differentiation in HL60 cells. The flavones apigenin and luteolin, the flavonols galangin, kaempferol and quercetin, the flavanone naringenin, the isoflavone genistein and the chalcone phloretin were used to clarify the relationship of the structure and the differentiation-inducing activity. The cell number and NBT reducing ability of HL60 cells treated with each flavonoid for 4 days were measured to study the effects of flavonoids on the growth and differentiation of HL60 cells. The isoflavone genistein induced NBT reducing ability, which is a marker of the differentiation into granulocytes and monocytes in HL60 cells, and inhibited cell growth. Genistein reduced the viable cell number to 7% of that of the dimethylsulfate-treated control cells and induced NBT reducing ability in 37% of viable cells at the concentration of 100 μM (Fig. 2). The effects of phloretin on the proliferation and differentiation of HL60 cells were less than those of genistein. The chalcone phloretin inhibited the cell growth to 28% of the control level and induced NBT reducing ability in 21% of cells at the concentration of 100 μM (Fig. 2). When HL60 cells were treated with 100 μM naringenin, the flavanone naringenin didn’t inhibit the cell growth nor induce NBT inducing ability, significantly (Fig. 2). Neither genistein, phloretin, nor naringenin had any effects on the growth and NBT reducing ability at the concentration of 1 μM, therefore the cells were treated with 10–100 μM flavonoids for further study. The incubation time was also extended to 5 days because even genistein induced NBT reducing ability in less than 40% of the cells. Apigenin, luteolin, quercetin, and galangin completely inhibited the cell growth of HL60 cells at the

![Chemical Structures](image)

**Fig. 1.** Structures and Chemical Classes of Flavonoids Used in this Study.

![Cell Growth and NBT Reducing Ability](image)

**Fig. 2.** Effects of Flavonoids on the Inhibition of Cell Growth and Induction of NBT Reducing Ability. HL60 cells (1 × 10⁶ cells/ml) were treated with DMSO (for control, ◯) or 1 μM (○), 10 μM (■), and 100 μM (●) of flavonoids for 4 days. NBT reducing ability was measured by the methods of Collins et al. And shown as the percentage of NBT-positive cells. Each value is the Mean ± S.D. of triplicate experiments. The Kruskal-Wallis test was done to detect the significant differences (p < 0.05) among all treatments including control (DMSO). Then the difference between control and each treatment was tested by the Wilcoxon rank sum test. *: p < 0.05
concentration of 100 μM. The viable cell numbers were reduced to 7–17% of the control by the treatment with 50 μM of the flavonoids (Fig. 3). The flavons apigenin and luteolin induced NBT reducing ability in about 30% of cells (Fig. 3). On the other hand, among flavonols only quercetin induced NBT reducing ability in 39% of cells (Fig. 3). Galangin and kaempferol also inhibited the growth of HL60 cells but didn't induce the NBT reducing ability in a dose-dependent manner (Fig. 3).

Apigenin induced not only the NBT reducing ability but also morphological change of HL60 cells into granulocytes. Segmented nuclei were observed in apigenin-treated HL60 cells (Fig. 4). Another flavone, luteolin, induced banded and segmented of nuclei like apigenin, but quercetin did not induce them (data of luteolin not shown). Genistein induced some metamyelocytic cells; the morphological differentiation was not as clear as that of the cells treated with apigenin. Phloretin induced NBT reducing ability in 21% of cells but scarcely induced morphological changes in the nuclei (Fig. 4). HL60 cells differentiated into monocytes showed large round nuclei and a high nucleus-to-cytoplasm ratio similar to undifferentiated control cells. Quercetin, phloretin, and naringenin may induce differentiation into monocytes.

α-Naphthyl butyrate esterase activity is a marker of the differentiation of promyelocytes into monocytes. Therefore to confirm the direction of differentiation in flavonoid-treated cells, we measured its activity in cells treated with apigenin, genistein, or phloretin and compared it with the NBT reducing ability. Apigenin and genistein completely inhibited the cell growth at concentrations of 60 μM and 80 μM, respectively. HL60 cells treated with 20 μM apigenin showed NBT reducing ability in 23% of the cells and α-naphthyl butyrate esterase activity in 22% of the cells (Fig. 5). NBT reducing ability induced by apigenin depended on the dose, but the α-naphthyl butyrate esterase activity did not (Fig. 5). Apigenin appeared to induce the differentiation of
HL60 cells into granulocytes but not into monocytes. Although genistein induced NBT reducing ability and α-naphthyl butyrate esterase activity in a dose-dependent manner, the ratio of α-naphthyl butyrate esterase positive cells to total cells was significantly less than that of NBT positive cells (Fig. 5). This result suggested that genistein induced both the granulocytic and monocytic differentiation of the cells.

Phloretin induced NBT reducing activity and α-naphthyl butyrate esterase activity in a dose-dependent manner and the ratio of their positive cells were not significantly different (Fig. 5). These results indicate that phloretin induced the differentiation of HL60 cells into monocytes.

### Discussion

As Constantinou et al. reported, genistein induced the NBT reducing ability, a marker of granulocytic- or monocytic differentiation, and α-naphthyl butyrate esterase activity, a marker of monocytic-differentiation of HL60 cells. Genistein appeared to induce both the monocytic- and granulocytic-differentiation of HL60 cells based on morphological observation, that is, partial induction of segmented nuclei. The results of increases in NBT reducing ability and α-naphthyl butyrate esterase activity in genistein-treated cells supported the hypothesis.

Apigenin was reported to induce differentiation in erythroleukemia cells. In our study, the flavones apigenin and luteolin strongly inhibited the growth and induced the granulocytic differentiation of HL60 myeloid leukemia cells. Among the flavonoids studied here, only the flavones induced morphologically maturated granulocytes. The flavonol quercetin and the flavanone naringenin were reported to induce the differentiation of erythrocytic cells. Quercetin inhibited the growth and induced the monocytic-differentiation of HL60 cells. It also induced α-naphthyl butyrate esterase activity as much as that of NBT reducing activity (data not shown).

The chalcone phloretin weakly inhibited the growth and induced monocytic differentiation of HL60 cells. Naringenin had almost no effect on differentiation and growth. Kinoshita et al. reported that galangin and kaempferol induced the differentiation of erythroleukemia cells and its differentiation-inducing activity was stronger than that of quercetin. However, the flavonols galangin and kaempferol, which have fewer hydroxyl group(s) in the B-ring than those of quercetin, did not induce the differentiation of HL60 cells. These results suggested that the pathways by which differentiation was induced in HL60 cells and mouse erythroleukemia cells were different.

Whether flavonoids induce the granulocytic or monocytic differentiation in HL60 cells depends on their structures. Flavonoids are known to modulate some physiological functions of cells, such as protein kinase C (PKC) activity, protein tyrosine kinase (PTK) activity, phosphatidylinositol turnover, and topoisomerase activities. Depending on the differences of their structures, flavonoids show different types of functions. Genistein is known to be a fairly specific inhibitor of PTK and topoisomerase. Phloretin specifically inhibits PKC activity and glucose transmembrane transport. Quercetin, kaempferol, and luteolin are potent inhibitors of PKC activity. Quercetin and kaempferol also significantly inhibit PTK activity as does genistein. Quercetin, apigenin, and luteolin are effective inhibitors of phosphatidylinositol 3-kinase activity. Quercetin, kaempferol, and apigenin strongly inhibited topoisomerase I. Furthermore, quercetin and kaempferol inhibited topoisomerase II activity.

Inhibition of PTK activity, suppression of phosphatidylinositol turnover, and inhibition of topoisomerase II activity were suggested as possible trigger of cell differentiation induced by flavonoids. Apigenin and luteolin may induce the granulocytic differentiation of HL60 cells through the inhibition of phos-
Flavonoid Induction of Granulocytic-/Monocyctic Differentiation of HL-60 Cells

Phatidylinositol because only the flavons induce the differentiation into granulocytes. However, quercetin, which induced monocytic differentiation, was also reported to strongly inhibit phosphatidylinositol 3-kinase activity strongly. Watanabe et al. reported that some combination of topoisomerases and protein kinase inhibitors induced the differentiation of mouse erythroleuke-
mia cells. On the other hand, PKC activity is known to modulate the induction of differentiation of leukemia cells as well as PTK and c-AMP-dependent protein kinase (PKA) activities. Flavonoids may induce granulocytic- or monocytic-differentiation due to the combination of these effects.

Flavonoids mostly induced differentiation in less than 50% of cells. The differentiation-inducing potency of flavonoids was not as strong as that of retinoic acid or sodium butyrate. However, in our previous study, we demonstrated that proanthocyanidins isolated from barley bran weakly induced the differentiation of HL60 cells and strongly potentiated the retinoic acid-induced granulocytic differentiation and sodium butyrate-in-
duced monocytic differentiation of HL60 cells. Flavonoids may also promote the induction of differentiation by some other inducers, such as retinoic acid and sodium butyrate, and support the differentiation of leu-
kenia and other blood cells under physiological condi-
tions.

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