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Growth arrest and apoptosis induced by quercetin is not linked to adipogenic conversion of human preadipocytes

Keiko Morikawa*, Chiharu Ikeda, Mitsuko Nonaka, Ikue Suzuki

Department of Nutrition, Division of Clinical Nutrition, Sagami Women's University, Sagamihara, Kanagawa 228-8533, Japan

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Abstract

Quercetin is involved in several biological activities including inhibition of cell growth and induction of apoptosis in cancer cells. However, it is unclear and unknown whether quercetin influences cell maturation. We examined the effect of quercetin on the growth and differentiation of human preadipocyte cells AML-I. Induced growth arrest of AML-I by quercetin was accompanied by the appearance of characteristics of apoptosis under the adipogenic stimulation by annexin V–fluorescein isothiocyanate staining method. A decrease of nuclear factor–κB and the antiapoptotic protein Mcl-1 and an increase of the proapoptotic protein Bad were observed in time-dependent fashion in the quercetin-treated cells compared with the vehicle-treated cells by Western blot analysis. Structure-related flavonoids, including rutin (quercetin-3-*O*-rutinoside) and quercitrin (quercetin-3-*O*-rhamnoside), did not have any cytotoxic effect on AML-I. Interestingly, exposure of AML-I to quercetin for 6 days increased the amount of cytoplasmic lipid droplets as well as the expression of fatty acid synthase and peroxisome proliferator–activated receptor γ proteins. These results suggested that apoptosis induced by quercetin was not linked to adipogenic conversion of preadipocytes.

1. Introduction

Flavonoids are naturally occurring phenolic compounds. The main sources of dietary flavonoids are in fruits, beverages, and, to a lesser extent, vegetables. It is well known that flavonoids are involved in a wide range of biological activities, including free radical scavenging, apoptosis induction, cancer chemoprevention, and protection from vascular disease [1]. The flavonoid quercetin affects cell cycle kinetics and proliferation and induces apoptosis in the cell culture [2,3].

Apoptosis is a highly regulated process that involves activation of a series of molecular events leading to cell death that is characterized by cellular morphological change, chromatin condensation, and more [4]. Two major pathways are sometimes referred to as the *extrinsic* and *intrinsic apoptotic pathways*. The extrinsic pathway can be induced by members of tumor necrosis factor family of cytokine receptors. These proteins recruit adapter proteins to their cytosolic death domains including Fas associating protein with death domain (FADD). The intrinsic pathway can be induced by the release of cytochrome *c* from mitochondria through various stimuli. The release of cytochrome *c* and subsequent apoptosis can be regulated by members of the Bcl-2 family of protein. These proteins are divided into 2 groups: proapoptotic proteins such as Bad and Bax and antiapoptotic proteins including Bcl-2, Bcl-X_L, and Mcl-1. The ratio between these 2 subsets helps determine, in part, the susceptibility of cells to a death signal [5]. Some of the signal transduction pathways, in which non–caspase-activating death domain proteins are involved, indirectly regulate apoptosis through effects on nuclear factor (NF)–κB. The NF-κB pathway is also a key mediator of genes involved in the control of cell proliferation and apoptosis. NF–κB activation can suppress cell death and protects the cell from apoptotic cascade [6,7]. Among NF-κB–inducible genes, several of them including antiapoptotic Bcl-2 family members block apoptosis.

Expansion of adipose tissue mass results from increased size of adipocyte cells and formation of new ones from precursor cells. The cellular and molecular events in the maturation of preadipocytes have been extensively studied using in vitro cell models such as a mouse preadipocyte cell line, 3T3-L1. 3T3-L1 cells can be induced to differentiate into mature adipocytes in cell culture [8-13]. The gene

* Corresponding author. Tel.: +81 42 749 4909; fax +81 42 749 4909.
E-mail address: morikawa_keiko@isc.sagami-wu.ac.jp (K. Morikawa).

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expression program leading to terminal adipocyte differentiation arises during and after the mitotic clonal expansion periods in 3T3-L1 preadipocytes [9,13,14]. However, other data indicate that this cell proliferation process is not a necessary step in the 3T3-L1 adipocyte differentiation process [8,10-12]. Although it remains controversial whether mitotic clonal expansion is a prerequisite for terminal differentiation, it is agreed that at least 2 families of transcription factors are involved via terminal differentiation by transactivation of adipocyte-specific genes. Members of the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator–activated receptor (PPAR) γ are key regulators in the adipogenesis process [14]. Exposure of preadipocytes to an adipogenic cocktail containing dexamethasone, isobutylmethylxanthine, and insulin induces C/EBP, which in turn activates PPAR-γ. In the terminal phase of differentiation, adipocytes markedly increase de novo lipogenesis. Fatty acid synthase (FAS) is a key metabolic enzyme catalyzing the synthesis of long-chain fatty acids from 2-carbon precursors. The activity, proteins, and messenger RNA level for enzymes involved in triglycerol metabolism including FAS increase at the terminal stage of adipocyte differentiation [14-16].

Recent studies have reported that some flavonoids inhibit the lipogenesis in chicken liver and cultured prostate cancer cells [17-20]. Epigallocatechin gallate (EGCG) inhibits growth of 3T3-L1 preadipocytes [21] or lipogenesis of 3T3-L1 adipocytes [22-24], whereas it increases the amount of cytosolic lipid droplets in NIH 3T3 and McA-RH7777 cells [25]. It has been reported that quercetin markedly inhibits lipogenesis in association with apoptosis in cancer cells [20], but it is uncertain whether quercetin affects the lipogenesis of adipocytes. In this study, we demonstrated that the growth arrest and apoptosis induced by quercetin did not affect adipogenic conversion of human preadipocytes.

2. Materials and methods

2.1. Reagents

Quercetin (3,3′,4′,5′,7-pentahydroxyflavone) and rutin (quercetin 3β-D-rutinoside, minimum 95% purity) were purchased from Sigma (St Louis, MO). Isoquercitrin (quercetin 3-D-glucoside) was synthesized in our laboratory.

Human antibodies for FADD, NF-κB (p65), α-tubulin, Bcl-2, Bcl-X_L, Mcl-1, Bad, Bax, FAS, and PPAR-γ and second antibodies for horseradish-peroxidase–conjugated mouse anti–rabbit immunoglobulin G were from Santa Cruz Biotechnology (Santa Cruz, CA); and goat anti–mouse immunoglobulin G was from Amersham Pharmacia Biotech (Arlington Heights, IL). Chemiluminescence detection system was supplied by Amersham Pharmacia Biotech.

2.2. Cell culture

The human preadipocyte cell line AML-I is possibly derived from a lineage of human bone marrow stromal cells as described previously [26]. Using the AML-I cell model, we investigated the effect of quercetin on the proliferation and differentiation of preadipocytes in vitro. The cell line has been maintained under standard culture conditions in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air and 1% penicillin-streptomycin in our laboratory. The cells were subcultured before leading confluency and the medium was replaced every 4 days because the serum component contained the factors facilitating cell differentiation from preadipocytes to adipocytes when they were confluent. Subconfluent cells obtained on day 3 after medium change were selected for the study.

2.3. Assay for cell growth

In the experiments, the cultures were switched to media containing 0.5 mmol/L methylisobutylxanthine (*M*), 0.5 μmol/L dexamethasone (*D*), and 1 mg/mL insulin (*I*) in dimethyl sulfoxide (DMSO) added to RPMI-1640/10% fetal bovine serum (*MDI* medium), unless otherwise mentioned. Flavonoids were reconstituted as 100 mmol/L stock solution in DMSO in a sterile condition, stored at −20°C, and resolved with MDI medium at the appropriate concentration upon use. Unless otherwise noted, *vehicle* refers to 0.1% DMSO in MDI medium, as final concentration of DMSO in quercetin solution was 0.1% in culture medium.

Subconfluent AML-I preadipocytes were seeded in 6-well plates at a density of 2.5×10^5 cells per milliliter in 5 mL MDI medium per well at day 1. Vehicle or quercetin at the concentration of 1, 10, or 100 μmol/L was added to the culture medium (MDI medium) at the time of plating. On days 2, 4, and 7 after plating, viable and dead cell numbers were measured by trypan blue dye exclusion test in a Neubauer hemocytometer.

2.4. Assay for apoptotic cell death

To determine whether the reduced viability in quercetin-treated cells was based on apoptosis or necrosis, the cells were stained using an annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma) and analyzed qualitatively and quantitatively by fluorescent microscopy. Briefly, 1×10^6 cells were washed twice, resuspended in phosphate-buffered saline (PBS), and stained with 5 μL of annexin V–FITC and 2.5 μL of propidium iodide according to the manufacturer's protocol, with minor modifications. Apoptotic cells were determined by fluorescent microscopy from a cytocentrifuged preparation. Cells at the early apoptotic stage were stained by annexin V–FITC. Live cells showed no staining by either propidium iodide or annexin V–FITC. Necrotic cells were stained by both propidium iodide and annexin V–FITC. At least 300 cells were observed on each slide, and the percentage of cells positive for annexin V–FITC was counted for each sample. A very small number of cultured AML-I cells began apoptosis in the MDI medium without quercetin

(spontaneous apoptosis), so the quercetin-specific apoptotic effect was estimated by the following formula.

$$\textit{Specific apoptosis}\ (\%) = \frac{(\textit{apoptosis by quercetin} - \textit{spontaneous apoptosis})}{(100 - \textit{spontaneous apoptosis})} \times 100$$

2.5. Morphological examination

2.5.1. Western blot analysis

Expression of apoptosis and differentiation-related proteins was examined by Western blot analysis. The AML-I cells were plated at a density of 2.5×10^5 cells per milliliter MDI medium in a 200-mm Petri dish with DMSO (0.1%) or quercetin (100 μmol/L) for indicated duration. Afterward, media were aspirated; and cells were washed with ice-cold PBS (pH 7.4). Cells were lysed in a lysis buffer, and Western blot analysis was performed using total cell lysate. For Western blot analysis, 100 μg of protein was loaded for each lane. Blots were incubated with the indicated primary antibodies and 1:25 000 or 1:50 000 horseradish peroxidase–conjugated second antibodies. All the primary antibodies were used at a final concentration of 2 μg/mL. Blots were then visualized with the chemiluminescence detection system as recommended by the manufacturer. To normalize the expression of the proteins, the relative expression levels of these proteins were evaluated on the basis of α-tubulin expression using CS analyzer software 2.0 (Atto Densitograph, Tokyo, Japan). The value of α-tubulin for the normalization of the protein expression was based on the assumption of no significant differences in the amount of α-tubulin per cell. Data were expressed as mean ± SD of the 3 different experiments.

2.6. Sudan black B staining and differentiation assay

After culture in the presence of 0.1% DMSO or quercetin at the concentration of 1, 10, or 100 μmol/L in MDI medium at 37°C for 6 days, a cytocentrifuged preparation of each sample was made; and cells were stained for lipid by incubating in the Sudan black B stain (0.18% Sudan black B made in 6% ethanol containing PBS) at 37°C for 60 minutes. Mature adipocytes were determined upon the presence of cytoplasmic lipid droplets under light microscopy. At least 300 cells were counted on each slide; and stage of adipocytes was evaluated according to the amount of cytoplasmic fat droplets that was measured semiquantitatively in accordance with the scoring method used for evaluating leukocyte alkaliphosphatase activity in the histochemical procedure, with some modifications as described [27]. Cells were classified into 3 categories: I, cells containing <5 droplets; II, cells containing unevenly distributed droplets; and III, cells containing densely distributed droplets.

2.7. Statistics

The data were expressed as mean ± SD. Student t test and analysis of variance (ANOVA) were used to determine significant differences. Values of $P < .05$ were considered significant.

3. Results

3.1. Inhibition of cell growth and viability in quercetin treatment

The AML-I cells were cultured in the presence of quercetin at concentrations of 1, 10, or 100 μmol/L (quercetin-treated cells) or 0.1% DMSO alone (vehicle-treated cells) in MDI medium for 7 days. Cell proliferation and viability were assessed using the trypan blue dye exclusion test and a hemocytometer.

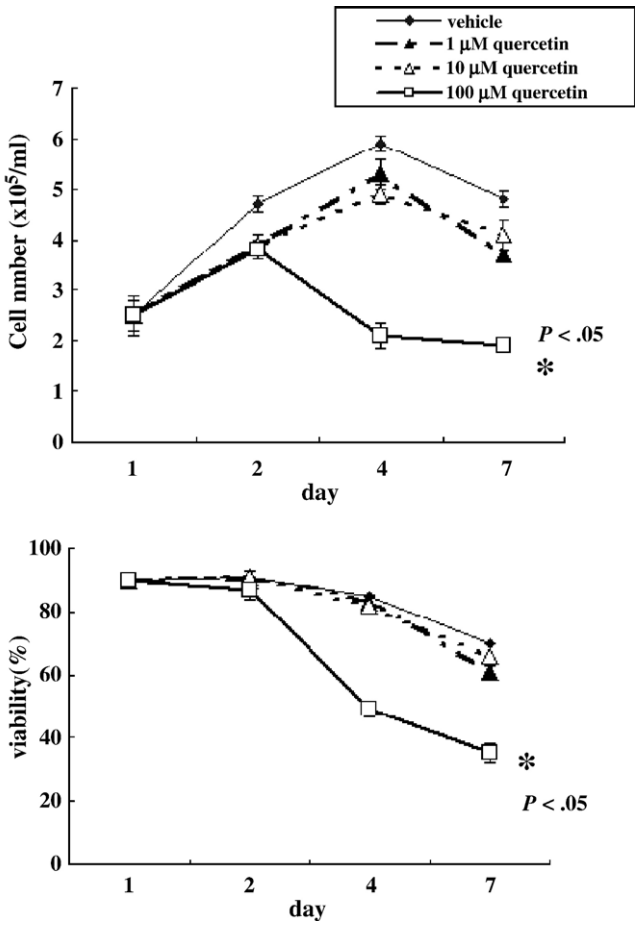

Fig. 1. Quercetin inhibits growth and viability of preadipocytes AML-I. AML-I preadipocytes were cultured in the presence of vehicle (0.1% DMSO) or 1, 10, and 100 μmol/L quercetin in MDI medium. On days 2, 4, and 7, cells were stained with trypan blue; and viable and dead cells were counted. Cell growth and viability are shown in the upper and lower panels, respectively. Data are represented as mean ± SD of triplicate cultures. A representative of 3 independent experiments is shown. Significant difference ($P < .05$) from control cells by Student t test.

The growth curve of cells cultured in the presence of vehicle demonstrated maximal proliferation at day 4 and gradual decline thereafter. In the presence of quercetin at concentrations between 1 and 10 μmol/L, little difference was observed in cell viability as well as cell proliferation between vehicle- and quercetin-treated cells in 7-day culture (Fig. 1). However, both growth and viability of AML-I were clearly suppressed in the presence of 100 μmol/L quercetin after day 4 (Fig. 1, upper and lower panels).

3.2. Induction of apoptosis in quercetin-treated cells

To determine whether quercetin-reduced cell number was caused by apoptosis, annexin V–FITC apoptosis assay was performed. As shown in Fig. 2, cells positive for annexin V–FITC were plentiful after quercetin treatment at day 4. When the apoptotic effect was calculated at days 4 and 6 as a percentage of quercetin-specific apoptosis as described earlier, quercetin-induced apoptosis was remarkably increased at the concentration of 100 μmol/L ($P < .05$) but not at 1 or 10 μmol/L (Fig. 3).

3.3. Influence of glycosylation on apoptotic effect

The effect of glycosylation on the biological functions of flavonoids is unclear. In this study, the cytotoxic effects on AML-I of 3 structure-related flavonoids including aglycone quercetin, glycone rutin (quercetin-3-*O*-rutinoside), and glycone quercitrin (quercetin-3-*O*-rhamnoside) were studied (Fig. 4). Concentrations of quercetin of 50 and 100 μmol/L suppressed cell viability ($P < .05$), but neither rutin nor quercitrin at a concentration of 100 μmol/L had this effect. These results suggested that attachment of sugar moiety might attenuate the apoptosis-inducing effect of quercetin.

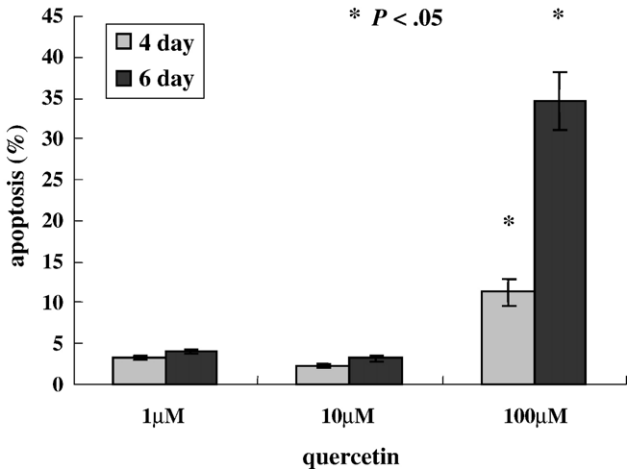

Fig. 3. Specific apoptosis of AML-I cells induced by quercetin. The AML-I cells were cultured with different concentrations of quercetin in MDI medium. On days 4 and 6, apoptotic cells were determined by annexin V–FITC assay as described in Materials and methods. Specific apoptosis (percentage) was calculated with the formula shown in Materials and methods. Experiments were repeated 3 times with similar results. Significant difference ($P < .05$) from control cells by ANOVA.

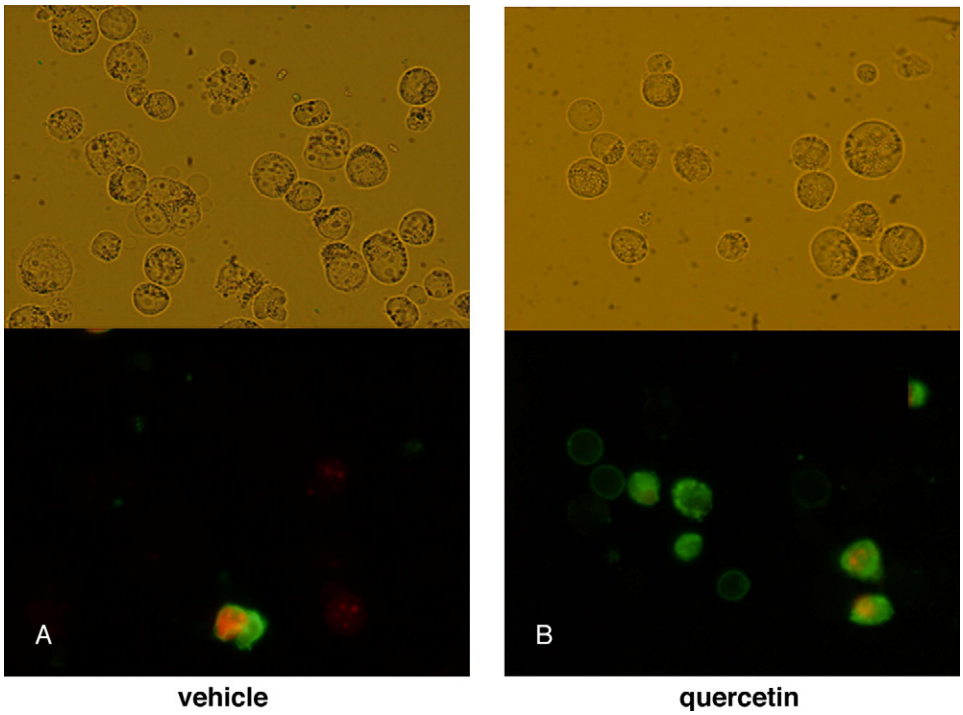

Fig. 2. Quercetin-induced apoptosis of AML-I cells. The AML-I cells were cultured in the presence of 0.1% DMSO (A) or 100 μmol/L quercetin (B) for 3 days. On day 4, cells were stained with annexin V–FITC and propidium iodide and were observed under fluorescence microscopy. Original magnification ×400 for all photographs. A representative of 4 independent experiments is shown.

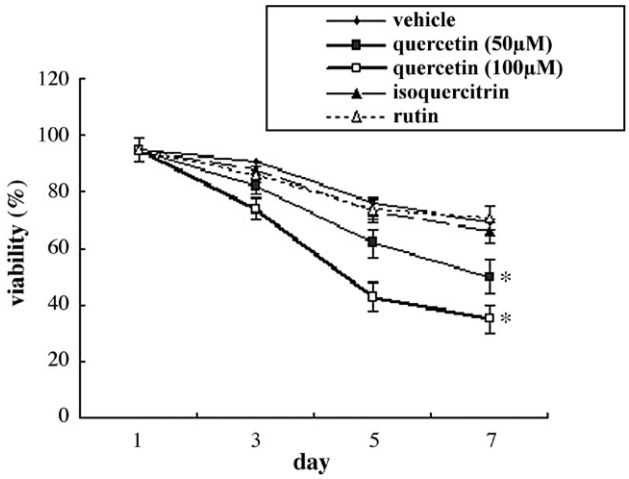

Fig. 4. Suppression of cell viability by quercetin treatment, but not by quercitrin or rutin. The AML-I cells were treated with vehicle, quercetin (50 or 100 μmol/L), isoquercitrin (100 μmol/L), or rutin (100 μmol/L). On days 3, 5, and 7, cell viability was detected by trypan blue dye exclusion test. Data are represented as mean ± SD of triplicate cultures. A representative of 3 independent experiments is shown. *Significant difference ($P < .05$) from control cells by ANOVA.

3.4. Effect of quercetin on the expression of apoptosis-related proteins

Expression levels of the apoptosis-related proteins FADD and NF-κB and several Bcl-2 family proteins such as Bcl-2, Bcl-X_L, Bad, Bax, and Mcl-1 were examined in the cell lysates obtained from cells treated with vehicle and quercetin 100 μmol/L at 2-, 6-, 24-, and 168-hour culture intervals by Western blot analysis (Fig. 5). Relative expression levels of FADD on the basis of α-tubulin expression did not differ between vehicle- and quercetin-treated cells at each interval. In contrast, the expression levels of NF-κB were reduced much more in quercetin-treated cells than in vehicle-treated cells after 6 days of culture as shown in Figs. 5 and 6. Relative expression levels of Bcl-2 and Bcl-X appeared to be increasing, but not significantly, in quercetin-treated cells. There were also no significant differences in expression levels of Bax at each interval. In contrast, levels of Bad decreased in vehicle-treatment but not in quercetin-treatment cultures. Conversely, Mcl-1_S and Mcl-1_L decreased in quercetin-treated but not in vehicle-treated cells (Figs. 5 and 6). These data indicated that quercetin induced an accumulation of proapoptotic Bad and loss of antiapoptotic NF-κB and Mcl-1 in a time-dependent manner. The amounts of NF-κB, Bad, Mcl-1_S, and Mcl-1_L proteins in vehicle- and quercetin-treated cells are displayed in Fig. 6.

3.5. Lipid productive activity by quercetin-treated cells

Next, we examined the effect of quercetin on the lipogenesis by AML-I cells. The cells were cultured for 3, 5, or 6 days in the presence of quercetin (100 μmol/L) or

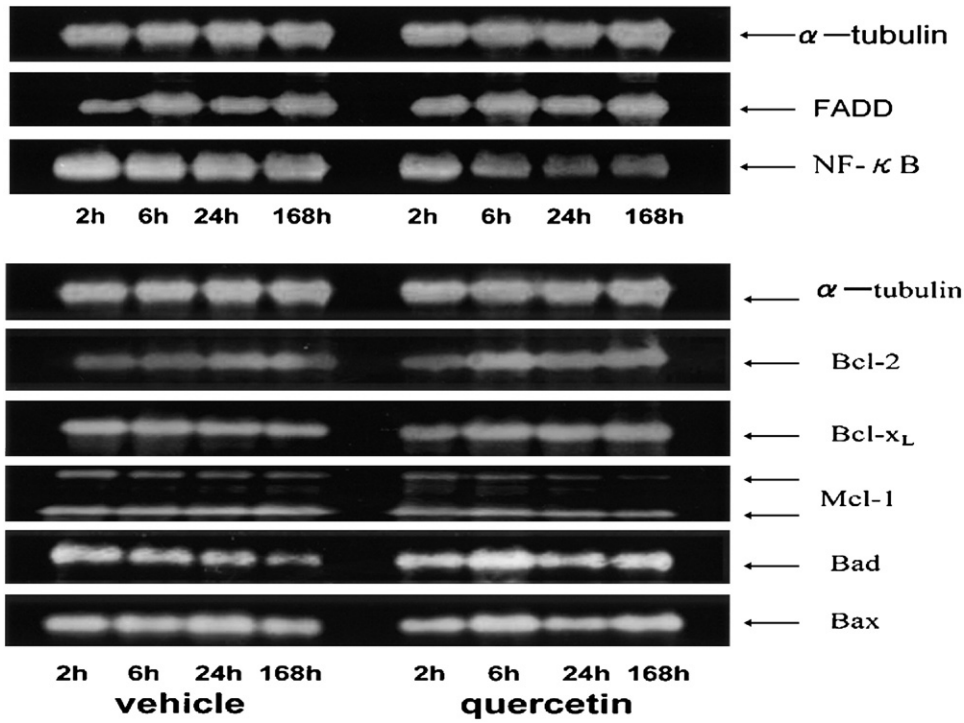

Fig. 5. Effect of quercetin (100 μmol/L) on the expression of apoptosis-related proteins. The AML-I cells were treated with 0.1% DMSO or 100 μmol/L quercetin in MDI medium for 2, 6, 24, and 168 hours. Afterward, cells were harvested and lysed for Western blot analysis as described in Materials and methods. Blots were incubated with mouse anti–α-tubulin, rabbit anti-FADD, rabbit anti–NF-κB, rabbit anti–Bcl-2, rabbit anti–Bcl-X_L, rabbit anti–Mcl-1, rabbit anti-Bad, mouse anti-Bax antibodies, and the second antibodies. Experiments were repeated 3 times with similar results.

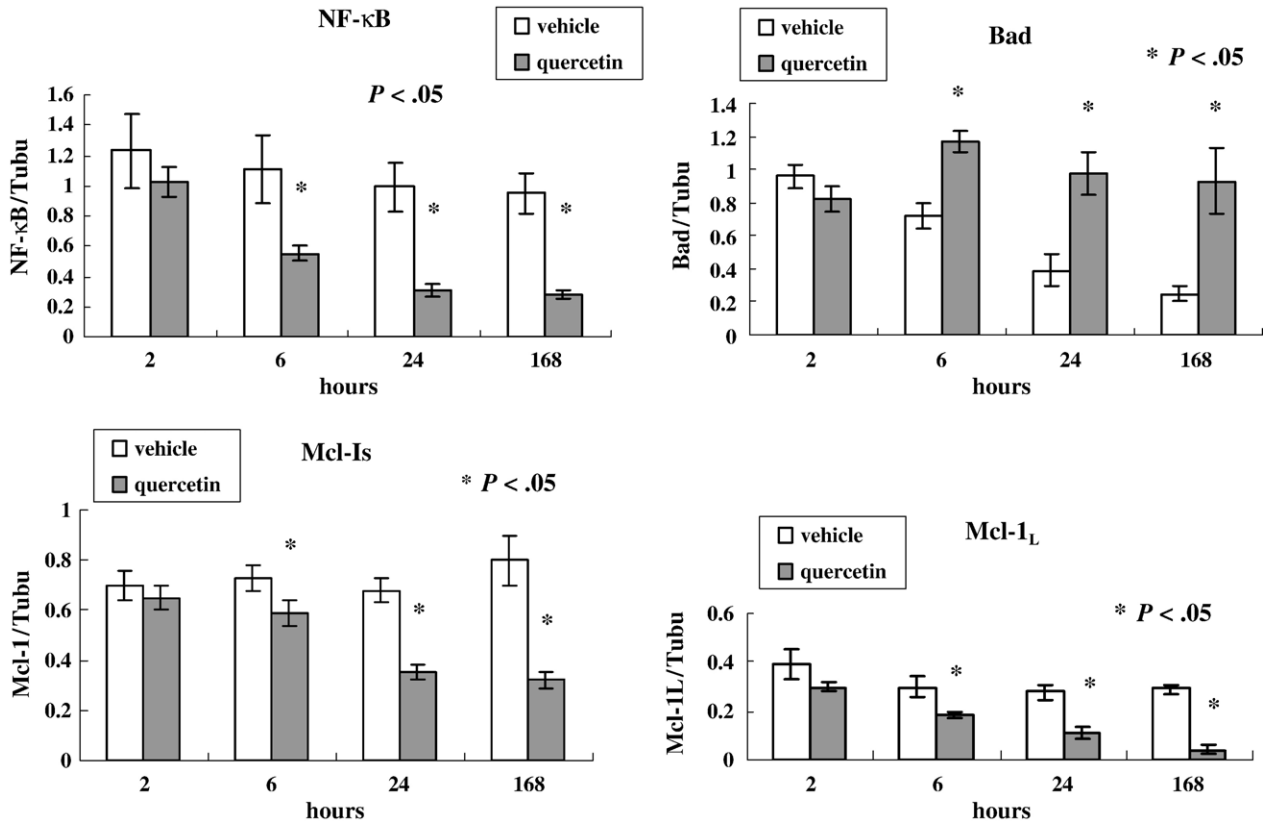

Fig. 6. The mean ± SD of normalized densities of bands corresponding to NF-κB, Bad, Mcl-1_S, and Mcl-1_L are shown in each graph. Significant difference ($P < .05$) from control cells by Student t test.

vehicle (0.01% DMSO) in MDI medium. Afterward, the cells were harvested; and the lipid droplet–containing cells were identified by Sudan black B staining under light microscopy. Although the number of lipid droplets varied among cells, they appeared to be plentiful in large adipocytes. The maturation of adipocytes was classified into 3 categories as described previously. Unexpectedly, cells treated with 100 μmol/L quercetin for 6 days contained more lipid droplets than those treated with vehicle. Cells classified as II and III were increased in a dose-dependent manner by quercetin treatment as shown in Fig. 7. As we have previously shown that AML-I cells can differentiate into adipocytes with an increase in cytoplasmic lipid droplets after adipogenic stimulation [26], these results suggested that quercetin might promote adipogenic conversion of preadipocytes.

3.6. Effect of quercetin on the expression of FAS and PPAR-γ proteins

Next, we examined the expression of PPAR-γ and FAS proteins in cells cultured in the presence or absence of quercetin, with or without adipogenic stimulation (MDI medium) for 7 days, by Western blot analysis. PPAR-γ is present at low levels in 3T3-L1 preadipocytes and is induced dramatically during adipocyte conversion during adipogenic stimulation [28]. FAS plays a central role in de novo lipogenesis during the differentiation of preadipocytes into adipocytes [15]. Our results displayed that small amounts of PPAR-γ and FAS proteins were expressed in cells cultured in standard medium without adipogenic stimulation (MDI [−]) for 7 days. The expression of PPAR-γ and FAS proteins was enhanced in the presence of adipogenic stimulators in culture medium (MDI [+]). Addition of 100 μmol/L quercetin to the MDI medium further increased the expression levels of these proteins as shown in Fig. 8. The depicted graph indicating the values of normalized densities of bands corresponding to FAS and PPAR-γ displayed that quercetin treatment was likely to promote the differentiation of AML-I preadipocytes into adipocytes.

4. Discussion

In the first part of this study, we demonstrated that 100 μmol/L quercetin inhibited proliferation and induced apoptosis in the human preadipocyte cell line AML-I. Annexin V–FITC assay displayed the characteristics of apoptosis in quercetin-treated cells. Loss of NF-κB and Mcl-1 and accumulation of Bad were associated with quercetin treatment as determined by Western blotting. These data verified the apoptosis-inducing effect of quercetin in preadipocytes.

Several biological activities of flavonoids are mediated through down-regulation of the NF-κB pathway [6,7,29].

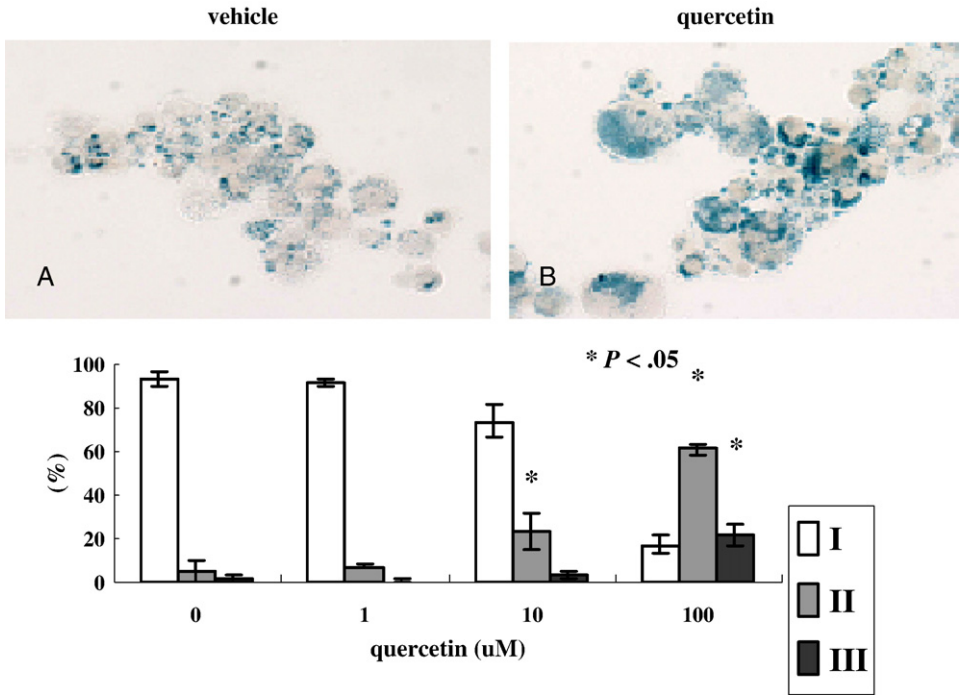

Fig. 7. Increase of lipid droplets in AML-I cells. The AML-I cells were cultured in the presence of vehicle or quercetin 1, 10, or 100 μmol/L with MDI medium. On day 6, cells were harvested; and cytoplasmic lipids were stained with Sudan black B, observed under light microscopy. Three hundred cells in each sample were counted and classified semiquantitatively into 3 categories as described in Materials and methods. Values are expressed as percentage, and a representative of 3 independent experiments is shown as a graph. Photographs were taken on day 6 of the culture (original magnification ×400). Significant difference ($P < .05$) from control cells by Student t test.

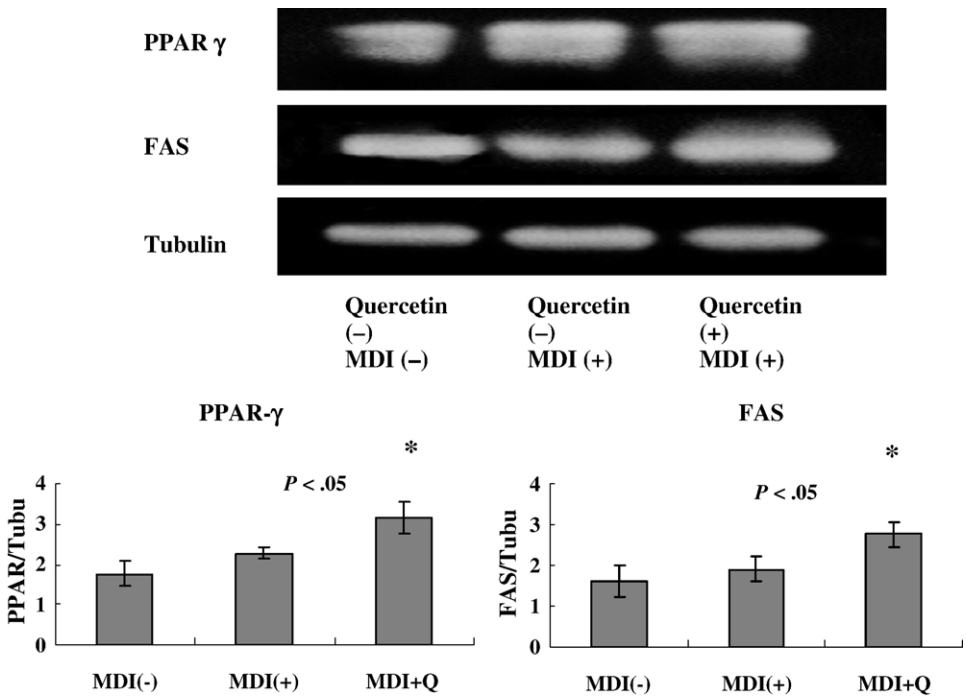

Fig. 8. Increased expression of PPAR-γ and FAS in AML-I cells by quercetin treatment. Cells were treated with vehicle (0.01% DMSO) or quercetin (100 μmol/L) in the absence (MDI [−]) or presence (MDI [+]) of MDI medium or quercetin (100 μmol/L) in the MDI medium. After 7 days of culture, cells were harvested and lysed for Western blot analysis as described in Materials and methods. Blots were incubated with rabbit anti-FAS, mouse anti–PPAR-γ, mouse anti–α-tubulin, and the second antibodies. Experiments were repeated 3 times with similar results. The values of normalized densities of bands corresponding to FAS and PPAR-γ in the presence or absence of quercetin or MDI medium are shown in each graph (lower part). Significant difference ($P < .05$) from control cells by Student t test.

Flavonoids including quercetin are known to block NF-κB activation and induce apoptosis in cancer cell lines [5,29]. Thus, the suppression of NF-κB protein in AML-I cells appears to be linked with the apoptotic effect of quercetin. Antiapoptotic and proapoptotic Bcl-2 family proteins are important regulators of cell survival and resistance to cytotoxic drugs. Antiapoptotic Bcl-2 family members exert their effect in part by inhibiting mitochondrial membrane permeabilization and maintaining mitochondrial homeostasis. The apoptosis inhibitory proteins Bcl-2 and Bcl-X_L exert their antiapoptotic effect, at least in part, by binding to Bax and Bad. Another Bcl-2 family protein, Mcl-1, is found in mitochondrial-enriched cell fractions and can heterodimerize with proapoptotic Bcl-2 family members such as Bax and Bak to prevent mitochondrial permeabilization. Loss of Mcl-1 is an apical event in the apoptotic cascade. In the present study, quercetin-induced apoptosis was associated with a decrease in the antiapoptotic protein Mcl-1 and an increase in the proapoptotic protein Bad, whereas other Bcl-2 family proteins including Bax, Bcl-2, and Bcl-X_L remained unchanged. Similar results have been reported for human leukemia cell line HL-60 and monocyte cell line THP-1 [30]. Ong et al [3] have reported that quercetin-induced apoptosis triggered an increase of both Bad and Bax in nasopharyngeal cancer–derived HK1 cells, whereas only Bad was observed to increase in nasopharyngeal cancer–derived CNE2 cells. The reason for this diversity is thought to be attributed to different sensitivities for the cytotoxic effect of quercetin on these cells [3]. Although we did not examine the phosphorylated status of Bad [31], the increase of Bad would indicate the accumulation of unphosphorylated type of Bad in the cytosol. Flavonoids exist as either simple or complex glycosides in many plants. Quercetin, rutin (quercetin-3-*O*-rutinoside), and quercitrin (quercetin-3-*O*-rhamnoside) are structurally related flavonoids and occur extensively in the human diet. Structural differences among them consist of rutinose in rutin and rhamnose in quercitrin at the C3 position of quercetin. Although reports on the effect of sugar moiety on the biological activity of quercetin have conflicted [32,33], the present study indicated that the glycosylated form of quercetin did not demonstrate the apoptotic effect. Shen et al [30] have also reported that quercetin, but neither rutin nor quercitrin, induces apoptosis in HL-60 cells. It is described that the bioavailability of quercetin and rutin from the digestive tracts to their plasma transport is less than 5% [34]. However, large uncertainties still remain in the absorption and metabolism of the flavonoids [1]. Taken together, apoptotic machinery by quercetin could be attributed to an accumulation of proapoptotic Bad protein and a loss of antiapoptotic Mcl-1 protein in preadipocytes.

In the latter half, we demonstrated an increase of PPAR-γ and FAS proteins as well as an accumulation of lipid droplets in quercetin-treated cells, which verified that quercetin stimulated the adipogenic conversion of AML-I preadipocytes. It has been thought that mitotic clonal expansion is a prerequisite for differentiation of 3T3-L1 preadipocytes into adipocytes [9,13]. The 3T3-L1 preadipocytes undergo approximately 2 rounds of mitotic clonal expansion, which just precedes the adipogenic gene expression program in the differentiation process in adipogenic culture medium [13]. However, some investigators have considered that cell proliferation is not a necessary step in the differentiation of 3T3-L1 cells. Bony et al [10] have shown that inhibition of mitogen-activated protein kinase (MAPK) accelerates the differentiation of subconfluent 3T3-L1 preadipocytes even in the absence of density-induced growth. In addition, a MAPK inhibitor, PD098059, blocks mitotic clonal expansion and accelerates adipogenic differentiation in confluent or postconfluent 3T3-L1 preadipocytes [8,11,12]. Thus, it remains unknown whether mitotic clonal expansion is a prerequisite for terminal differentiation or if the cell proliferation and differentiation are regulated by 2 separate signal transduction pathways. In our data, quercetin appeared to stimulate the differentiation process, despite growth arrest, of preadipocytes. The mode of action of quercetin is very similar to that of PD09805. Apoptotic cell death did not occur during overnight PD098059 treatment in 3T3-L1 cells in the study of Bony et al, and we could not observe the apoptosis of AML-I cells within 24 hours of quercetin treatment. The apoptotic effect of quercetin was detected after 48 hours of the culture and was maximal between 72 and 96 hours after the culture initiation. Enhancement of differentiation without mitotic clonal expansion has been reported in human adipocyte precursor cells [35], myoblasts [36], and rat fetal brown adipocytes [37]. These results suggest that the differentiation process is not related with the mitotic clonal expansion in preadipocytes. The effect of EGCG on 3T3-L1 cells may imply a subject of controversy on the adipocyte conversion of preadipocyte. EGCG is reported to induce growth arrest and apoptosis in 3T3-L1 preadipocytes [21]. Others indicate that EGCG blocks adipogenesis and increases apoptosis in mature 3T3-L1 adipocytes [22,23], but has no effect on either viability or apoptosis of 3T3-L1 preadipocytes [22]. Furuyashiki et al [24] have described that EGCG does not affect cell growth by insulin signal pathway, but suppresses adipocyte differentiation by down-regulation of PPAR-γ2 and C/EBPα in confluent 3T3-L1 preadipocytes. It has been determined that the mitotic clonal expansion of 3T3-L1 preadipocytes is induced by insulin and by neither 1-isobutyl-3-methyl-xanthine nor dexamethasone in the MDI medium [11]. In contrast, differentiation of 3T3-L1 preadipocytes into adipocyte is induced by 1-isobutyl-3-methyl-xanthine and dexamethasone in the MDI medium [11]. Furthermore, recent study demonstrates that EGCG increases formation of cytoplasmic lipid droplets and decreases secretion of apolipoprotein B-100 very low-density lipoprotein in NIH3T3 cells and McA-RH7777 cells [25]. The mechanism mediating the quercetin-induced lipogenesis is uncertain, but inhibition of MAPK pathway may be one of the possible mechanisms because several

studies indicate that loss of MAPK activation inhibits DNA synthesis and accelerates adipocyte differentiation [8,10-12]. Further investigation is necessary to elucidate the effect of quercetin on the adipocyte biology.

Although the lipogenesis usually indicates the differentiation of preadipocytes into adipocytes, it can be observed without a true adipogenic conversion in a special culture condition. Fink et al [38] have reported that mesenchymal stem cell forms an adipocyte-like feature with cytoplasmic lipid inclusion during 72 hours of the culture under an atmosphere of 1% oxygen. Such cells express neither PPAR-γ nor ADDI/SREBP1c adipogenic regulators. Although the culture in our study was not undertaken in such hypoxic condition, we examined the expression of PPAR-γ and FAS proteins in the cells to verify the adipocyte conversion. PPAR-γ is largely adipocyte specific and is expressed at low levels in preadipocytes and at maximal levels in mature adipocytes [14], and FAS is expressed at high levels during the terminal phase of adipocyte differentiation [14]. Enhancement of the expression of PPAR-γ and FAS by quercetin treatment suggested the adipogenic conversion of AML-I preadipocytes.

Nelson-Dooley et al [39] have described that apoptotic process in adipocytes may be an important target for the treatment of obesity and osteoporosis. If the processes of differentiation and proliferation are regulated separately in preadipocytes, the block of the differentiation process may be necessary for their treatment, too.

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