Journal of Cellular Biochemistry 89:1044–1055 (2003)

Differential Apoptosis-Inducing Effect of Quercetin and its Glycosides in Human Promyeloleukemic HL-60 Cells by Alternative Activation of the Caspase 3 Cascade

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Abstract Flavonoids were demonstrated to possess several biological effects including antitumor, antioxidant, and anti-inflammatory activities in our previous studies. However, the effect of glycosylation on their biological functions is still undefined. In the present study, the apoptosis-inducing activities of three structure-related flavonoids including aglycone quercetin (QUE), and glycone rutin (RUT; QUE-3-O-rutinoside), and glycone quercitrin (QUI; QUE-3-O-rhamnoside) were studied. Both RUT and QUI are QUE glycosides, and possess rutinose and rhamnose at the C3 position of QUE, respectively. Results of the MTT assay showed that QUE, but not RUT and QUI, exhibits significant cytotoxic effect on HL-60 cells, accompanied by the dose- and time-dependent appearance of characteristics of apoptosis including an increase in DNA ladder intensity, morphological changes, apoptotic bodies, and an increase in hypodiploid cells by flow cytometry analysis. QUE, but not RUT or QUI, caused rapid and transient induction of caspase 3/CPP32 activity, but not caspase 1 activity, according to cleavage of caspase 3 substrates poly(ADP-ribose) polymerase (PARP) and D4-GDI proteins, and the appearance of cleaved caspase 3 fragments being detected in QUE- but not RUT- or QUI-treated HL-60 cells. A decrease in the anti-apoptotic protein, Mcl-1, was detected in QUE-treated HL-60 cells, whereas other Bcl-2 family proteins including Bax, Bcl-2, Bcl-XL, and Bag remained unchanged. The caspase 3 inhibitor, Ac-DEVD-FMK, but not the caspase 1 inhibitor, Ac-YVAD-FMK, attenuated QUE-induced cell death. Results of DCHF-DA assay indicate that no significant increase in intracellular peroxide level was found in QUE-treated cells, and QUE inhibited the H_2O_2-induced intracellular peroxide level. Free radical scavengers *N*-acetyl-cysteine (NAC) and catalase showed no prevention of QUE-induced apoptosis. In addition, QUE did not induce apoptosis in an mature monocytic cell line THP-1, as characterized by a lack of DNA ladders, caspase 3 activation, PARP cleavage, and an Mcl-1 decrease, compared with those in HL-60 cells. Our experiments provide evidence to indicate that the addition of rutinose or rhamnose attenuates the apoptosis-inducing activity of QUE, and that the caspase 3 cascade but not free radical production is involved. J. Cell. Biochem. 89: 1044–1055, 2003.

Key words: quercetin; quercitrin; rutin; apoptosis; caspase 3; PARP

INTRODUCTION

The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and apoptosis. However, apoptosis is often inhibited or attenuated in tumors which have a higher cell proliferation rate. Therefore, one of the attractive methods for cancer chemoprevention

Abbreviations used: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribosyl) polymerase; Bcl-2, B-cell lymphoma 2; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; QUE, quercetin; RUT, rutin; QUI, quercitrin; DEVD, Asp–Glu–Val–Asp; YVAD, Tyr–Val–Ala–Asp.

Grant sponsor: The National Science Council of Taiwan; Grant numbers: NSC89-2320-B-038-054, NSC 90-2320-B-038-027, NSC 91-2320-B-038-040.

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Received 24 February 2003; Accepted 31 March 2003
DOI 10.1002/jcb.10559

or chemotherapy is dietary or pharmaceutical production to induce death of tumor cells through apoptosis. Apoptosis, or programmed cell death, 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 apoptotic bodies which are associated with DNA cleavage into ladders [Chen et al., 1996, 2002b; Yu et al., 2000]. Several pathways have been described to regulate apoptosis during development, tumorigenesis, and chemical treatments [Budd, 2002]. Previous studies indicated that cells from a variety of human malignancies have a decreased ability to undergo apoptosis in response to certain physiological stimuli [Barry et al., 1990; Nagata and Golstein, 1995]. The expression of several genes has been demonstrated to be critical for the regulation of apoptosis such as caspase cascades and Bcl-2 family proteins. Human caspases 1–10 have been described, and a previous study demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis [Huigsloot et al., 2001]. Caspase 9 is an apoptosis initiator which is activated by binding with Apaf-1, a homolog of CED-4, to induce its oligomerization [Kanuka et al., 1999; Rodriguez and Lazebnik, 1999]. Activated caspase 9 then cleaves and activates executioner caspase 3, which exists as an inactive pro-caspase 3 in the cytoplasm and is proteolytically activated by multiple cleavages of pro-caspase 3 to generate the cleaved fragments in cells undergoing apoptosis [Dong et al., 1999; Arita et al., 2000]. After caspase 3 activation, some specific substrates for caspase 3 such as poly(ADP-ribose) polymerase (PARP) and D4-GDI proteins are cleaved which are important for the occurrence of apoptosis. PARP is required for DNA repair, and activated caspase 3 cleaves PARP at Asp 216 to generate the 85 and 31 kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis [Ray et al., 2001; Soldani et al., 2001]. And, D4-GDI is a negative regulator of the ras-related Rho family of GTPases, and activation of Rho GTPases promoted cytoskeletal and membrane changes associated with apoptotic cells. Activated caspase 3 cleaves D4-GDI into 23- and 5-kDa fragments, and activates Rho GTPases to produce apoptotic morphological changes [Danley et al., 1996; Krieser and Eastman, 1999]. Bcl-2 family proteins modulate the occurrence of apoptosis and tumorigenesis [Su et al., 2001]. Members of the Bcl-2 family proteins can be divided into two sub-families, one is anti-apoptotic including Bcl-2, Mcl-1, and Bcl-XL proteins and the other is pro-apoptotic including Bax, Bcl-Xs, and Bad. Induction of pro-apoptotic Bcl-2 family proteins and inhibition of anti-apoptotic family proteins have been detected in apoptosis induced by chemicals [Lee et al., 2001].

Flavonoids are a group of natural products, and exert several biological functions such as apoptosis-inducing activity, free radical scavenging activity, and anti-tumorigenic activity [Lin et al., 1997; Liang et al., 1999; Ko et al., 2002; Lee et al., 2002; Sang et al., 2002]. Although flavonoids have been extensively studied, application of flavonoids in the treatment of human diseases is still uncommon because of undefined in vivo metabolites, higher effective concentrations, and poor absorptive activity after oral intake. Glycosylation commonly occurs in the metabolism of flavonoids, and increases their hydrophilic properties by the addition of sugar moieties into the structure. The effect of glycosylation on the biological function of flavonoids is still undefined, and our previous study suggested that glycosylation attenuated the anti-inflammatory activity of flavonoids in macrophages [Chen et al., 2001]. In order to demonstrate the effect of glycosylation on the apoptosis-inducing activity of flavonoids, three structurally related flavonoids, including quercetin (QUE) (aglycone), rutin (RUT) (glycone; QUE + rutinose), and quercitrin (QUI) (glycone; QUE + rhamnose) which posses a different glycosylation status, were studied to identify their apoptosis-inducing activities in human promyeloleukemia HL-60 cells. Results of the present study suggest that adding rutinose or rhamnose attenuates the apoptosis-inducing activity of QUE, and that the caspase 3 cascade but not ROS production might be involved in the differential apoptotic mechanism.

MATERIALS AND METHODS

Cell Culture and Chemicals

HL-60 human promyelocytic leukemia cells and THP-1 human mature monocytic cells were obtained from American Type Culture Collection (ATCC). HL-60 cells were grown at 37°C in RPMI 1640 containing 10% heat-inactivated

fetal bovine serum in an atmosphere containing 5% CO_2. Exponentially HL-60 cells were exposed to drugs for the indicated time periods. The plates for HL-60 cells were purchased from Gibco (GIBCO/BRL, Grand Island, NY). The colorigenic synthetic peptide substrates for caspase 3-like proteases (Ac-DEVD-pNA) and for caspase 1 (Ac-YVAD-pNA) were purchased from Calbiochem, La Jolla CA. The inhibitors for caspase-3-like proteases (Ac-DEVD-FMK) and for caspase 1 (Ac-YVAD-FMK) were purchased from Calbiochem. Propidium iodide, QUE, RUT, and QUI were obtained from Sigma Chemical Co., St. Louis, Missouri, MO. Antibodies for PARP, caspase 3, and D4-GDI detection in Western blotting were obtained from IMGENEX, San Diego, CA. Antibodies for detecting Bcl-2 family proteins were purchased from Santa Cruz Laboratories, Santa Cruz, CA. DCHF-DA was obtained from Molecular Probe, Eugene, OR.

Cell Viability

Cell viability was assessed by MTT staining as described by Mosmann [1983]. Briefly, HL-60 cells were plated at a density of 10^6 cells/ml into 24-well plates and treated with different concentrations of indicated compounds for 8 h. At the end of treatment, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (10 mg/ml) was added, and cells were incubated for a further 4 h. Cell viability was obtained by scanning using an ELISA reader (Molecular Devices, Sunnyvale, CA) with a 600-nm filter.

Determination of ROS Production

ROS production was monitored by flow cytometry using DCFH-DA [Chen et al., 1998]. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. In the present study, HL-60 cells were treated with QUE (40 μM) for 2 h. Then, QUE-treated cells were washed twice with PBS to remove the extracellular emodin, and DCHF-DA (100 μM) with or without H_2O_2 (200 μM) was added for an additional 1 h. Green fluorescence was excited by using an argon laser and was detected using a 525-nm band pass filter by flow cytometry analysis.

Western Blots

Total cellular extracts (30 μg) were prepared and separated on 8% SDS–polyacrylamide minigels for PARP detection and 12% for SDS–polyacrylamide minigels for caspase 3, cleaved D4-GDI, Bcl-2 family, and α-tubulin protein detection, and then were transferred to Immobilon polyvinylidenedifluoride membranes (Millipore Corp., Bedford, MA). The membrane was incubated overnight at 4°C with 1% bovine serum albumin at room temperature for 1 h and then incubated with indicated antibodies for a further 3 h at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for 1 h. Protein was visualized by incubating with the colorimetric substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) as described in our previous paper [Chen et al., 1996].

DNA Gel Electrophoresis

Cells (10^6/ml) under different treatments were collected, washed with PBS twice, and then lysed in 100 ml of lysis buffer (50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) for 3 h at 56°C and treated with 0.5 mg/ml RNase A for an additional 1 h at 56°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point argarose, 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gel containing 0.1 μg/ml ethidium bromide. The agarose gels were subjected to 50 V for 90 min in TBE buffer. The gels were observed and photographed under UV light.

Activities of Caspase 3 and Caspase 1 (ICE) Proteases

After different treatments, cells (10^6/ml) were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colori-

metric substrates including Ac-DEVD-pNA for caspase 3/CPP32 and Ac-YVAD-pNA for caspase 1 at 37°C for 1 h. Alternative activity of caspase 3 or caspase 1 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm [Chen et al., 2002a].

Flow Cytometry Analysis

Trypsinized cells (10^6/ml) were washed with ice-cold PBS and fixed in 70% ethanol at −20°C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5% Triton X-100/PBS at 37°C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide for 10 min. Fluorescence emitted from the propidium–DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson, Franklin Lakes, NJ).

Statistics

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A P value of < 0.01 or < 0.05 was regarded as indicating a significant difference.

RESULTS

Induction of Apoptosis by QUE, but not RUT or QUI

Glycosylation has been demonstrated to occur during metabolism of flavonoids; however, the effect of glycosylation on the apoptosis-inducing activity of flavonoids is still undefined. Here, the apoptosis-inducing effects of three structurally related flavonoids with different glycosylation status including aglycone QUE, glycone RUT, and glycone QUI were investigated. The chemical structures of QUE, RUT, and QUI are shown in Figure 1. Arrows indicate the different groups among these three flavonoids. When HL-60 cells were treated with various concentrations of each indicated compound (20, 40, and 80 μM) for 8 h, the viability of HL-60 cells was significantly reduced in the presence of QUE, but not in RUT- or QUI-treated HL-60 cells (Fig. 2B). DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of HL-60 cells. To characterize cell death induced by QUE, the integrity of genomic DNA, the occurrence of apoptotic bodies, and the ratio of hypodiploid cells were examined. In the analysis of DNA

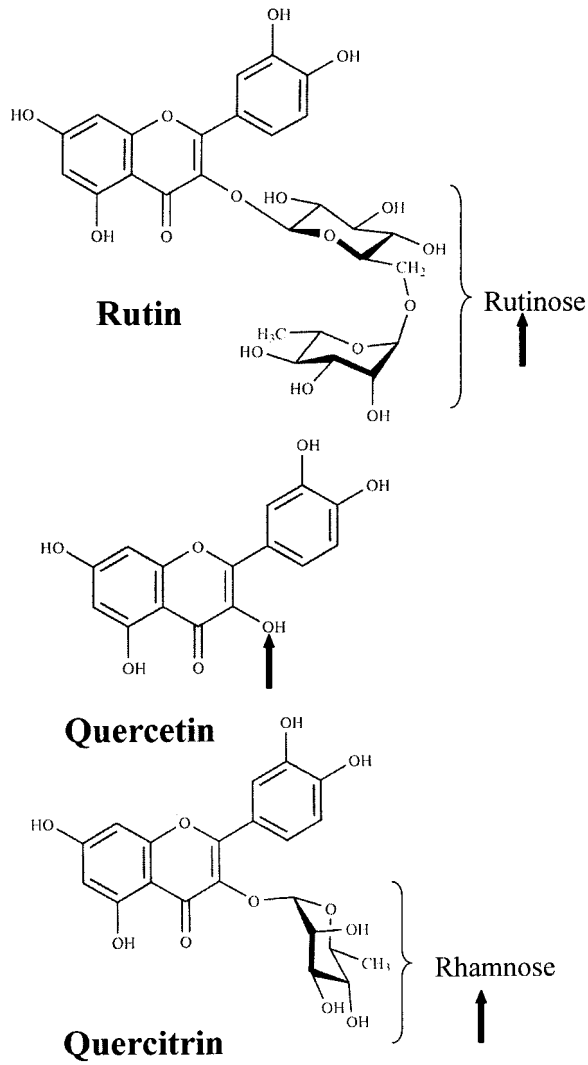

Fig. 1. Structurally related flavonoids including quercetin (QUE), rutin (RUT), and quercitrin (QUI). RUT and QUI contain a rutinose and rhamnose at the C3 position of QUE. The arrow indicates differences among QUE, RUT, and QUI.

integrity by agarose electrophoresis, QUE treatment caused the digestion of genomic DNA into ladders in concentration- and time-dependent ways, associated with a decrease in intact DNA (Fig. 2A). However, with RUT and QUI, even at 40 μM, no significant amount of DNA ladders were found. In the same part of the experiment, morphological changes and the ratio of hypodiploid cells were examined under microscopic observation and by flow cytometric analysis, respectively. QUE, but not RUT or QUI, induced the occurrence of apoptotic bodies under microscopic observation, accompanied by an increased ratio of hypodiploid cells under flow cytometric analysis (Fig. 3). No obvious DNA ladders, apoptotic bodies, or hypodiploid cells

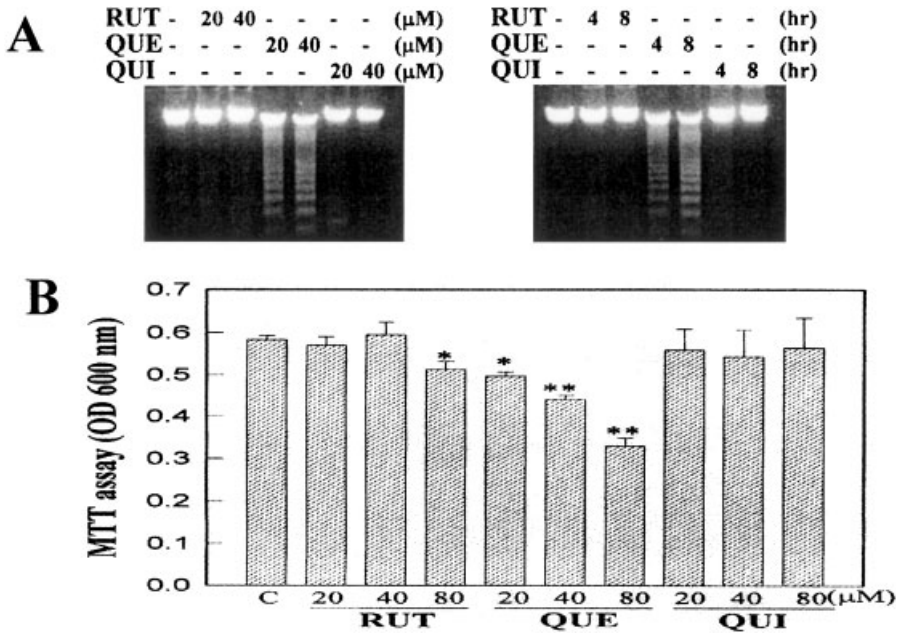

Fig. 2. Analysis of cell viability and DNA integrity in QUE-, RUT-, and QUI-treated HL-60 cells by MTT assay and agarose electrophoresis. **A**: (**left panel**) HL-60 cells were treated with different concentrations (20 or 40 μM) of QUE, RUT, or QUI for 8 h. **Right panel**: HL-60 cells were treated with QUE, RUT, or QUI (40 μM) for 4 and 8 h. DNA in cells was extracted and electrophoresed through a 1.8% agarose gel and visualized by staining with ethidium bromide. **B**: HL-60 cells were plated into 24-well plates for 24 h and were then treated with different concentrations of the indicated compound (20, 40, or 80 μM) for a further 8 h. MTT was added to the medium for an additional 4 h. The viability of cells was detected by measuring the absorbance at a wavelength of 600 nm. Each value is presented as the mean ± SE of three independent experiments. $*P < 0.05$, $**P < 0.01$ significantly different from the control, as analyzed by Student's *t*-test.

were detected in DMSO-treated HL-60 cells. These data demonstrate that QUE, but not RUT and QUI, is an apoptosis inducer in HL-60 cells.

Stimulation of Caspase 3-Like Activities, not Caspase 1-Like Activities, in QUE-Induced Apoptosis

Previous data indicated that the induction of caspase activities is an essential event in apoptosis. Caspase 1 and caspase 3 have been shown to be involved extensively in the apoptotic process. To detect the enzymatic activity of caspase 1 and 3 in QUE-, RUT-, and QUI-treated HL-60 cells, two colorimetric substrates, Ac-DEVA-pNA for caspase 3-related activities and Ac-YVAD-pNA for caspase 1-related activities, were used in this study. As illustrated in Figure 4, QUE induced a dramatic increase in DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells. In the contrast to QUE, neither the DEVD-specific nor YVAD-specific enzyme activity was stimulated in RUT- or QUI-treated cells. It suggests that activation of caspase 3, but not caspase 1, activity exhibited in QUE-induced apoptosis. To determine if the activation of the caspase 3-like protease is necessary for apoptosis induced by QUE, caspase inhibitors including the caspase 3-like protease inhibitor, Ac-DEVD-FMK, and the caspase 1-like protease inhibitor, Ac-YVAD-FMK, were used to block intracellular protease, and QUE-induced DNA ladders were analyzed by agarose electrophoresis. Results in Figure 4 (right panel) show that the caspase 3-like inhibitor, Ac-DEVD-FMK (20 and 40 μM), inhibited the occurrence of DNA ladders induced by QUE. However, Ac-YVAD-FMK, an inhibitor of caspase-1-like activity, showed no obvious effect at the same concentrations. These data demonstrate that activation of caspase 3-like activity is involved in QUE-induced apoptosis.

QUE-Induced Apoptosis Independent of ROS Production in HL-60 Cells

Free radical-producing and -scavenging activities have been described as biological effects of flavonoids. In order to further demonstrate if ROS production is involved in apoptosis induced by QUE, the intracellular peroxide level was determined by measuring the fluorescence intensity of DCF, and changes in intracellular peroxide levels were determined by flow cyto-

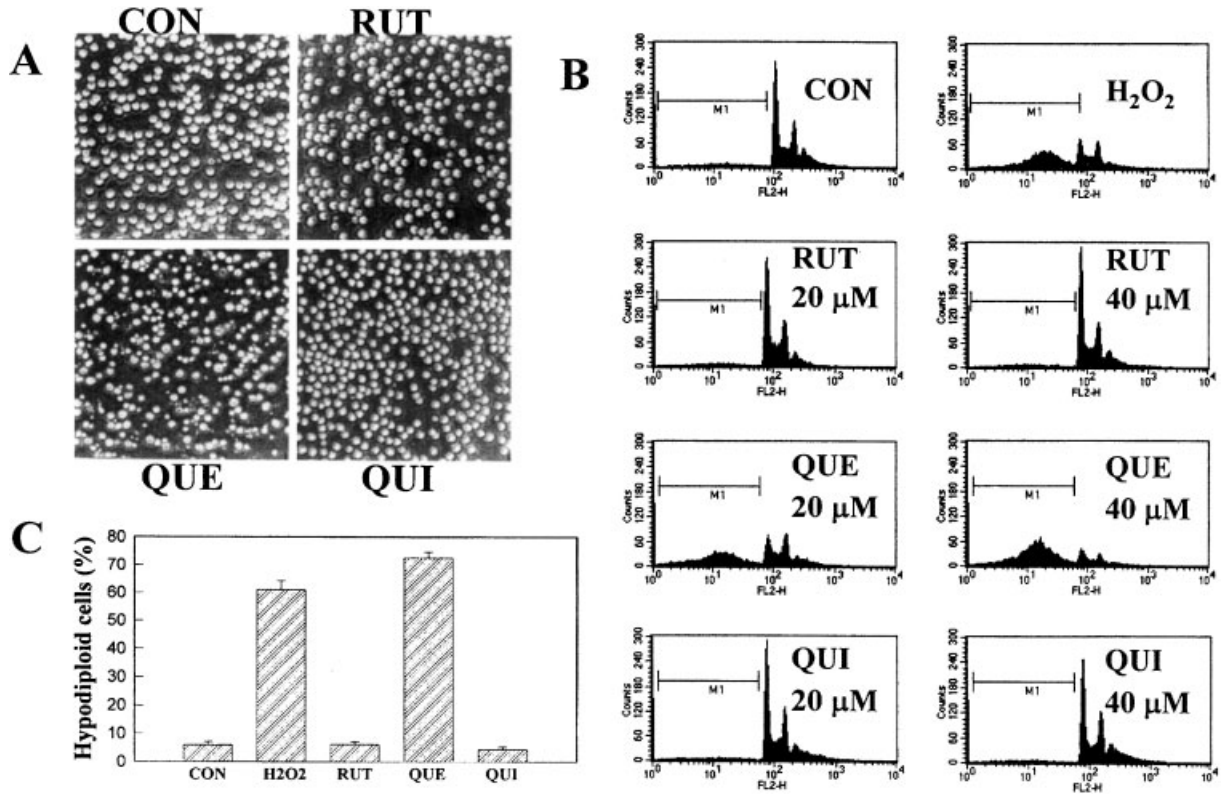

Fig. 3. Appearance of chromosomal condensation, hypodiploid cells, and apoptotic bodies in QUE- but not RUT- or QUI-treated HL-60 cells. **A**: Occurrence of apoptotic bodies and chromosomal condensation in HL-60 cells was detected in 40 μM QUE- but not RUT- or QUI-treated HL-60 cells under light microscopic observation. **B**: Induction of hypodiploid cells in 20 or 40 μM QUE- but not RUT- or QUI-treated HL-60 cells. HL-60 cells were treated with or without QUE, RUT, and QUI (20 or 40 μM) for 8 h, and the appearance of hypodiploid cells was detected by flow cytometry using PI staining. A representative of the result of flow cytometry analysis is shown. **C**: The M1 apoptotic value in (B) is expressed as the mean ± SE of three independent experiments by flow cytometry analysis.

metric analysis. The results in Figure 5 show that QUE alone produced no change in endogenous intracellular peroxide levels in the absence of H_2O_2 treatment. Adding H_2O_2 (200 μM) into the medium with DCHF-DA elevated the intracellular fluorescence intensity to 2,309.7 ± 21.7 and is described as a positive control here. Prior treatment with QUE (40 μM) for 2 h were significantly attenuated following a H_2O_2 (200 μM)-induced increase in intracellular peroxide level in HL-60 cells ($^{**}P < 0.01$). The free radical scavengers, *N*-acetyl-cysteine (NAC) and catalase (CAT), were used in the present study, and results in Figure 5B show that neither NAC nor CAT showed a preventive effect on QUE-induced DNA fragmentation, whereas NAC and CAT significantly inhibited H_2O_2-induced DNA fragmentation as described in our previous paper [Chen et al., 2002a,b]. These data demonstrate that QUE-induced apoptosis is independent of ROS production.

Involvement of PARP and D4-GDI Cleavage, the Caspase 3 Protein Procession, and a Decrease in Mcl-1 Protein in QUE- but not RUT- or QUI-Treated Cells

Activation of caspase 3 leads to the cleavage of a number of proteins, two of which are PARP and D4-GDI, another hallmark of apoptosis. Results in Figure 6 show that exposure of HL-60 cells to QUE but not RUT or QUI caused the degradation of the 116-kDa PARP into 85-kDa fragments and the production of cleaved D4-GDI protein (23 kDa) in a concentration-dependent manner. This was associated with the protein procession of caspase 3 brought about by its cleavage, represented here as a decline in its pro-level and the appearance of cleaved fragments on the Western blot. However, RUT and QUI showed no obvious effects on PARP, D4-GDI, or caspase 3 cleavage in HL-60 cells. Bcl-2 family proteins act as important regulators of

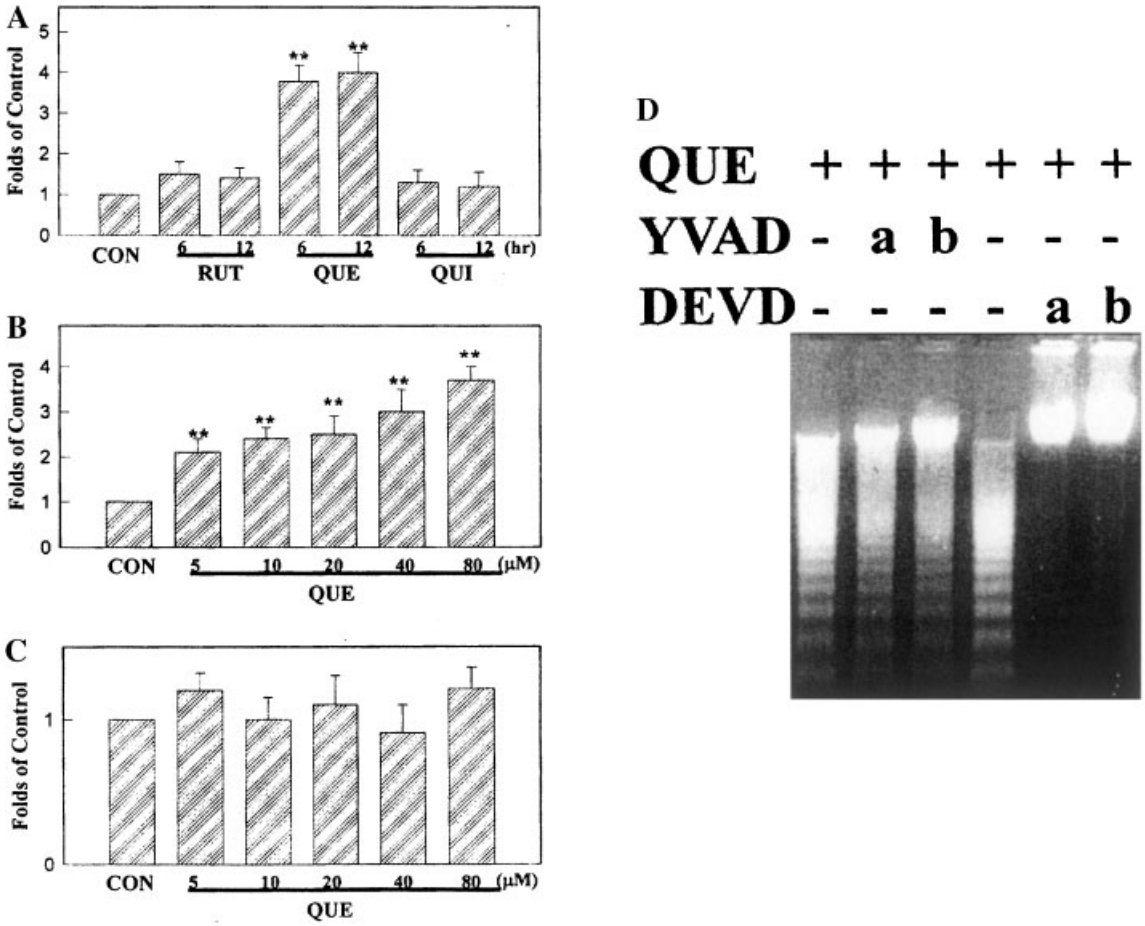

Fig. 4. Activation of caspase 3 but not caspase 1 activity in QUE (40 μM)-treated HL-60 cells. **A**: HL-60 cells were treated with QUE, RUT, or QUI (40 μM) for 6 and 12 h, and cells were harvested and lysed in lysis buffer. The enzymatic activity of caspase 3-like proteases was determined by incubation with the specific colorigenic substrate, Ac-DEVD-pNA. **B**: HL-60 cells were treated with different doses of QUE (5, 10, 20, 40, or 80 μM), and caspase 3 activity was detected using Ac-DEVD-pNA as a substrate. **C**: Cells were treated as described in (B) and caspase 1 activity in cells was measured by using Ac-YVAD-pNA as a substrate. **D**: The caspase 3 peptidyl inhibitor, Ac-DEVD-FMK (DEVD), but not the caspase 1 peptidyl inhibitor, Ac-YVAD-FMK (YVAD), inhibited QUE-induced apoptosis. Cells were treated with different doses of (a: 20 μM; b: 40 μM) indicated inhibitors for 1 h, followed by QUE (40 μM) treatment for a further 8 h. The integrity of DNA in cells was examined as described in Figure 2.

apoptosis and are located upstream of caspase activation. In QUE-treated HL-60 cells, a decrease in the Mcl-1 protein was detected in a dose-dependent manner. In contrast to decreases in the Mcl-1 proteins, Bcl-2, Bcl-XL, Bag, and Bax, proteins remained unchanged in QUE-treated HL-60 cells (Fig. 6A). No obvious change was detected in the expression of Bcl-2 family proteins in RUT- or QUI-treated HL-60 cells.

QUE Showed no Significant Apoptosis-Inducing Activity in the Mature Monocytic Cell Line THP-1

HL-60 is a poorly differentiated promyelocytic leukemia cells. In order to identify if QUE exhibits differential apoptosis-inducing activities between poorly differentiated and well-differentiated cells, another leukemia cell line THP-1 is used in the present study. THP-1 is a mature monocyte, and has been described as a well-differentiated leukemia cell line. Investigating the effect of QUE in HL-60 and THP-1 cells was performed in the present study. Results in Figure 7 show that QUE induced DNA ladders, caspase 3 activation, and PARP and D4-GDI cleavage in poorly differentiated leukemia HL-60 cells, but not in mature monocytic THP-1 cells. This indicates the differential apoptotsis-inducing effects of QUE in poorly differentiated cells (HL-60) and well-differentiated cells (THP-1).

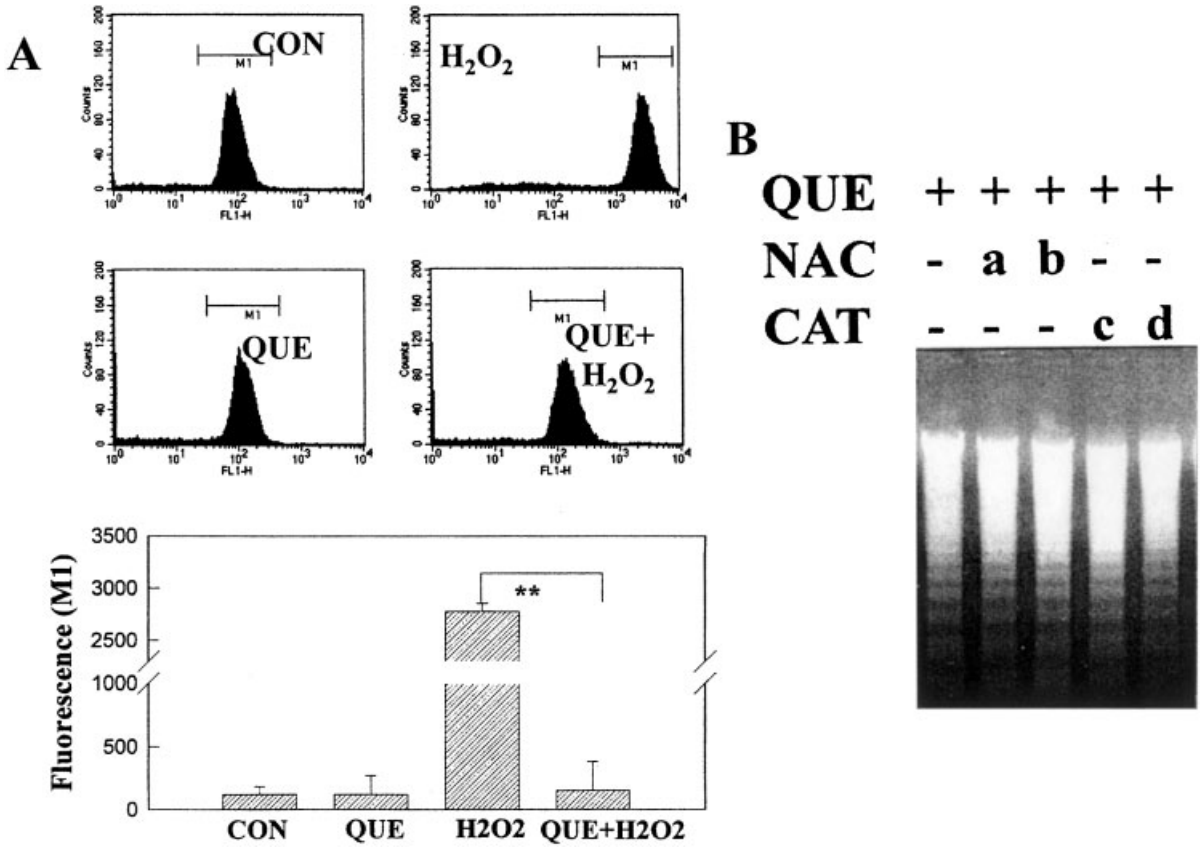

Fig. 5. Effect of QUE on intracellular peroxide levels by DCHF-DA assay. **A**: HL-60 cells were treated with QUE (40 μM) for 2 h, followed by addition of DCHF-DA (100 μM) with or without H_2O_2 (200 μM) for an additional 1 h. The fluorescence intensity of cells was measured by flow cytometry analysis. The **upper panel** is representative of the result of flow cytometry analysis, and the **lower panel** is the value of fluorescence presented as the mean ± SE of three independent experiments. $^{**}P < 0.01$ significantly different from the H_2O_2-treated group, as analyzed by Student's *t*-test. **B**: NAC and catalase showed no preventive effect on QUE-induced DNA ladders. Cells were treated with NAC (a: 10 mM; b: 20 mM) or CAT (c: 200 U/ml; d: 400 U/ml) for 1 h followed by QUE (40 μM) treatment for a further 8 h. The integrity of DNA was analyzed as described in Figure 2.

DISCUSSION

Several biological functions of flavonoids have been identified, whereas there is a lack of evidence to support the relationship between glycosylation and apoptosis-inducing activity. In the present study, we demonstrate that QUE is able to induce apoptosis in the human leukemia cell line, HL-60, however neither QUE glycosides RUT and QUI, even at a dose of 80 μM, affected the viability of cells. The induction of apoptosis by QUE is consistent with the activation of apoptotic machinery including activating the caspase 3 cascade, but is independent of ROS production. Additionally, QUE did not induce apoptosis in well-differentiated monocytic THP-1 cells. Results of the present study suggest that: (1) the addition of rutinose or rhamnose into QUE attenuates the apoptosis-inducing activity of QUE; (2) differential caspase 3 activation exists in QUE-, RUT-, and QUI-treated cells; (3) QUE-induced apoptosis is independent on its pro-oxidant activity; and (4) QUE preferentially induces apoptosis in poorly differentiated leukemia HL-60 cells, but shows less cytotoxicity to well-differentiated monocytic THP-1 cells.

Flavonoids exist as either simple or complex glycosides in many plants, and humans are estimated to consume approximately 1 g flavonoids/day. QUE, RUT (QUE-3-O-rutinoside). QUE, RUT (QUE-3-O-rutinoside), and QUI (QUE-3-O-rhamnoside) are structurally related flavonoids and exist extensively in the human diet. Structural differences among them consist of a rutinose in RUT and a rhamnose in QUI at the C3 position of QUE. QUE can be derived from RUT through hydrolyzation by glucosidase in the gastrointestinal tract [Manach et al., 1997], and it has a wide range of biological

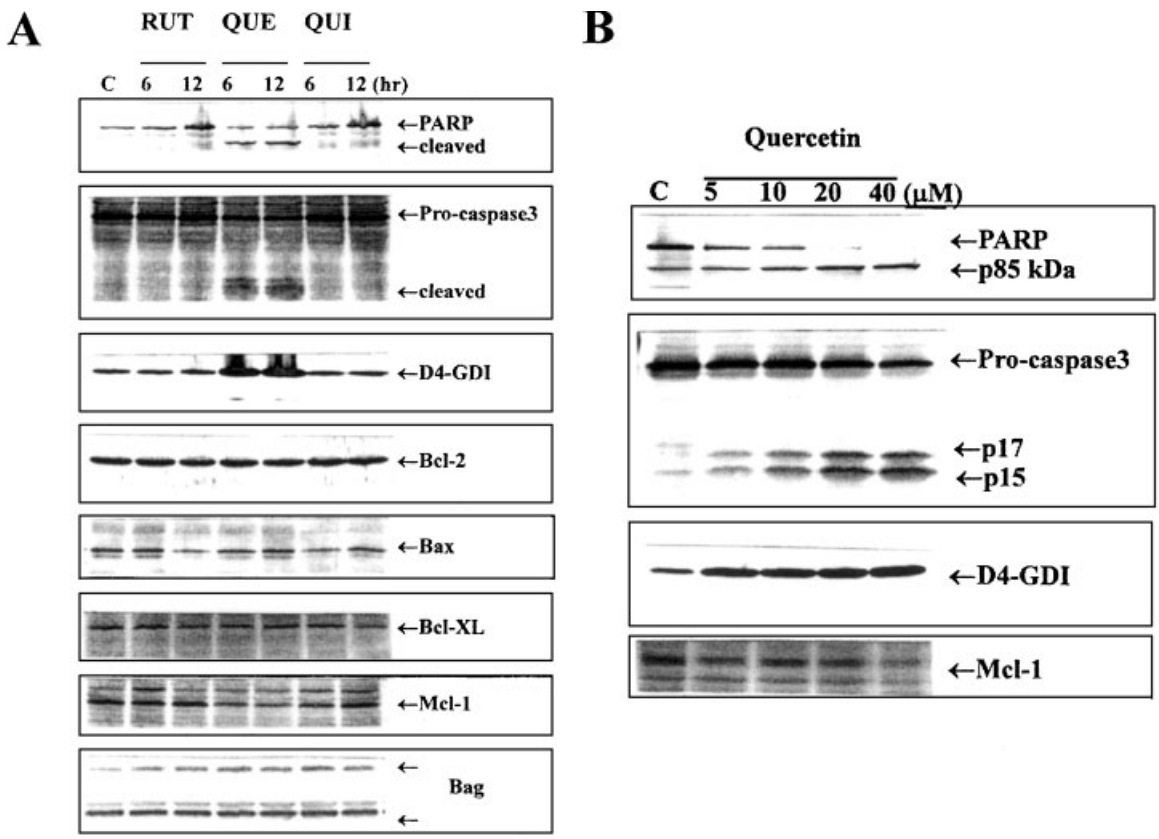

Fig. 6. Induction of caspase 3 protein processing, PARP and D4-GDI protein cleavage, and a decrease in Mcl-1 protein in QUE- but not RUT- or QUI–treated HL-60 cells. **A**: HL-60 cells were treated with, 40 μM QUE, RUT, or QUI for 6 or 12 h. Bcl-2 family proteins including Bcl-2, Bcl-XL, Bax, and Mcl-1, as well as PARP and D4-GDI cleavage and induction of caspase 3 protein procession were analyzed by Western blotting as described in ''Materials and Methods''. **B**: Dose-dependent response of QUE in the caspase 3 protein procession, PARP and D4-GDI cleavage, and decreasing Mcl-1 protein. HL-60 cells were treated with different doses of QUE (5, 10, 20, or 40 μM) for 12 h, and expression of the indicated genes was analyzed by Western blotting.

activities such as inhibition of Na^+/K^+-ATPase, protein kinase C, tyrosine kinase, etc. [Kang and Liang, 1997]. Both RUT and QUI can be hydrolyzed by cell-free extracts of human fecal cultures of QUE to exhibit mutagenic effects [Macdonald et al., 1983]. Furthermore, QUE-3-glucoside can be absorbed more effectively in the small intestine than QUE itself [Morand et al., 2000]. Ioku et al. [1998] suggested that dietary flavonoid glucosides were primarily hydrolyzed and liberated as aglycones in the jejunum [Ioku et al., 1998]. In contrast, Crespy et al. [2002] reported that QUE could be absorbed in the stomach, but QUE glycosidic forms such as RUT were not absorbed [Crespy et al., 2002]. These data suggested that conversion of QUE glycoside to QUE occurs both in vivo and in vitro, and the glycosylation status of QUE related to its in vivo absorption efficiency. However, the effect of glycosylation on the biological activity of QUE is still unclear. Kim et al. [1999] reported that QUE transformed from QUI showed more potent cytotoxicity against tumor cell lines than did QUI. Our recent studies demonstrated that QUE, but not RUT, showed significant inhibitory activity against lipopolysaccharide-induced NO production by blocking iNOS gene expression in macrophages [Chen et al., 2001; Shen et al., 2002]. In the present study, we found that QUE, but not RUT or QUI, induced apoptosis in cells. These results indicate that the addition of rutinose or rhamnose to C3 of QUE inhibits the apoptosis-inducing activity of QUE.

Apoptosis induction by QUE has been shown in previous studies [Wei et al., 1994; Schroeter et al., 2001], however, the apoptotic mechanism of QUE has not been confirmed. Caspase cascade has been shown to be involved in the action of apoptosis, and at least ten caspases have been described. Caspase 3 is an executioner caspase, and exists in the cytoplasm as an

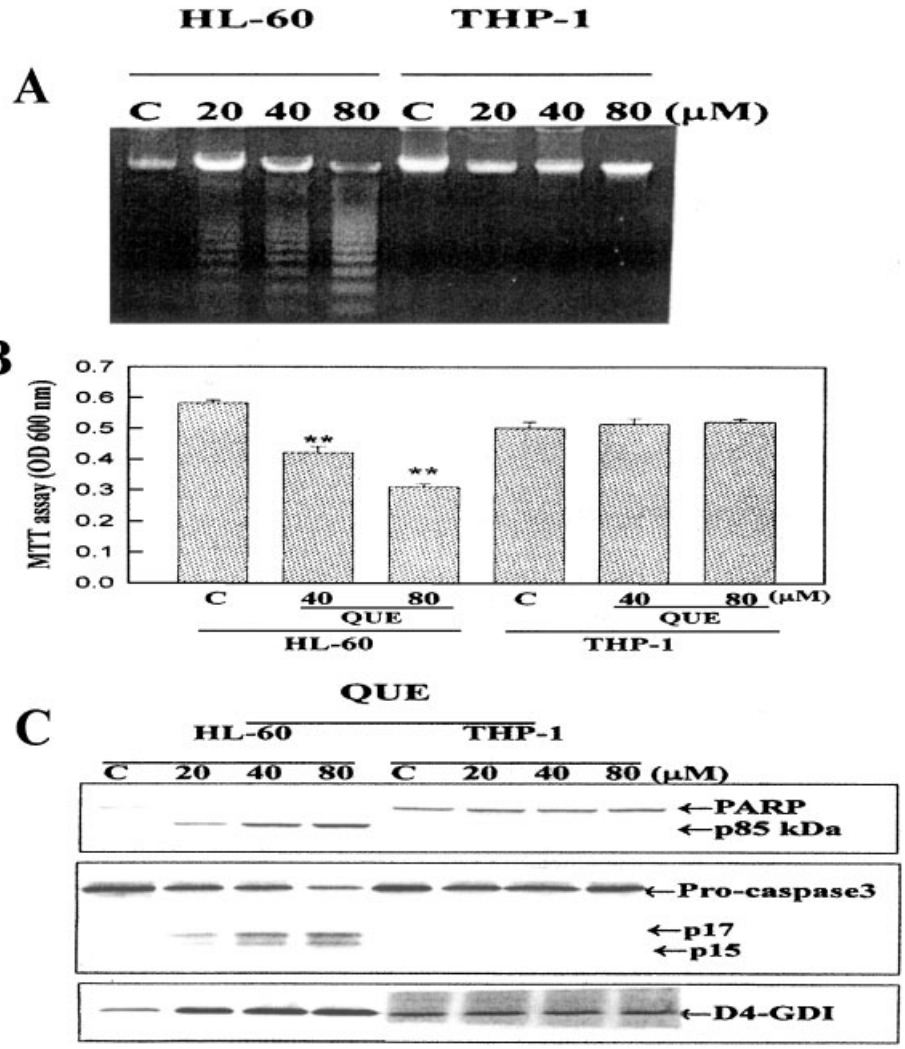

Fig. 7. Differential apoptosis-inducing effect of QUE in poorly differentiated leukemia HL-60 cells and well-differentiated mature monocytic THP-1 cells. **A**: QUE showed significant apoptosis-inducing activity in HL-60, but not in THP-1, cells. Both cells were treated with QUE (20, 40, or 80 μM) for 8 h, and the integrity of DNA in cells was analyzed. **B**: MTT assay for cellular viability as described in Figure 2 was performed to identify the differential cytotoxicity of QUE in HL-60 and THP-1 cells. **C**: QUE did not induce the caspase 3 protein procession, or PARP or D4-GDI cleavage in THP-1 cells, compared with those in HL-60 cells. Both cells were treated with different doses (20, 40, or 80 μM) QUE for 12 h, and the expression of indicated genes was examined as described in Figure 6.

inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its precursor 32 kDa to generate the 20/11 or 17/11 kDa active forms in apoptotic cells. In addition, Bcl-2 family proteins have been demonstrated to be involved in the process of apoptosis, and pro-apoptotic, including Bax, Bak, and Bcl-Xs, and anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-XL, and Mcl-1 have been identified. Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins participated in the process of apoptosis. So far, the roles that the caspase cascade and Bcl-2 family proteins play in QUE-induced apoptosis are still undefined. Results of this study demonstrate a decrease in Mcl-1 protein, and activation of caspase 3 (not caspase 1) activity in QUE- but not RUT- or QUI-treated HL60 cells.

QUE has been described as possessing the prooxidant and antioxidant activities, and a previous study indicated that QUE, but not RUT or QUI, was a mutagenic flavonol through its ROS production activity [Bjeldanes and Chang, 1977]. Therefore, it is interesting to investigate

if ROS are involved in the differential apoptosis-inducing activities of QUE, RUT, and QUI. In the present study, we found that QUE, RUT, and QUI itself did not alter intracellular peroxide levels; however QUE, but not RUT or QUI, inhibited H_2O_2-induced peroxide production (Fig. 5 and data not shown). Both NAC and CAT inhibited H_2O_2-induced apoptosis, whereas neither of them showed any inhibition of QUE-induced cell death. This suggests that ROS production is involved in the differential apoptosis-inducing activities of QUE, RUT, and QUI.

In conclusion, results of the present study provide scientific evidence to support that the addition of rutinose or rhamnose attenuating the apoptosis-inducing activity of QUE, and the involvement of the caspase 3 (not caspase 1) cascade but not ROS production. More evidence demonstrating the effect of other glycosides substitutions such as glucoside or complex glucosides on the apoptosis induction of QUE needs to be further evaluated.

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