Quercetin induces FasL-related apoptosis, in part, through promotion of histone H3 acetylation in human leukemia HL-60 cells

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Abstract. Quercetin, a naturally occurring flavonoid abundant in fruits and vegetables, has been demonstrated as a multi-potent bioflavonoid with great potential for the prevention and treatment of cancer. Apoptosis is thought to be an important response to most chemotherapeutic agents in leukemia cells. However, the underlying mechanism of induction of apoptosis by quercetin involving epigenetic regulation is poorly understood. In the present study, by evaluation of fragmentation of DNA, poly (ADP-ribose) polymerase (PARP) and procaspases, we found that quercetin was able to induce apoptosis of human leukemia HL-60 cells in a dose-dependent manner. Quercetin triggered the extrinsic apoptosis pathway through activation of caspase-8 and induction of Bid cleavage, Bax conformation change and cytochrome *c* release. Furthermore, quercetin induced Fas ligand (FasL) expression involving activation of the extra-cellular signal-regulated kinase (ERK) and Jun N-terminus kinase (JNK) signaling pathways. In addition to activation of c-Jun, quercetin increased histone H3 acetylation which resulted in the promotion of the expression of FasL. Quercetin exhibited potential for the activation of histone acetyltrans-ferase (HAT) and the inhibition of histone deacetyltransferase (HADC), both of which contributed to histone acetylation. However, only the activation effect on HAT was associated with the ERK and JNK pathway. These results demonstrated that quercetin induced FasL-related apoptosis by trans-activation through activation of c-jun/AP-1 and promotion of histone H3 acetylation in HL-60 cells.

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Introduction

Apoptosis plays a critical role in normal development, homeostasis and in the defense response against pathogens. Inappropriate suppression or activation of apoptosis can lead to a variety of diseases. This kind of cell death is thought to be an important response to most chemotherapeutic agents in leukemia cells. Apoptosis is mediated through two major pathways, the extrinsic and intrinsic pathways, which both lead to the activation of caspases. The extrinsic pathway is triggered at the plasma membrane by the activation of the death receptor (Fas) and subsequently the activation of caspase-8. In some cells, caspase-8 directly activates down-stream effector caspases such as caspase-3 while in other cell types caspase-8 mediates apoptosis via the proteolytic cleavage of the pro-apoptotic Bid protein. Following Bid cleavage and its translocation to mitochondria, truncated Bid (t-Bid) induces oligomerization and conformational changes in Bak and Bax, resulting in the release of cytochrome *c* and the procession of effector caspases (1,2). The intrinsic pathway is triggered by various apoptotic stress signals and is characterized by mitochondrial dysfunction, which leads to the release of mitochondrial pro-apoptotic factors from their intermembrane space into the cytosol. The release of cyto-chrome c from mitochondria represents a critical event in initiating the activation of the caspase cascade. This occurs through its interaction with Apaf-1, and the subsequent processing and activation of the cell death protease, caspase-9. Activated caspase-9 triggers the catalytic maturation of effecter caspases such as caspase-3, which triggers oligonucleosomal DNA fragmentation (3,4).

Cell responses to apoptotic-inducing agents have been associated with the inactivation of survival kinases and the activation of apoptotic kinases. One of the most relevant aspects in the regulation of apoptosis is the involvement of mitogen-activated protein kinases (MAPKs), a family of proline-directed serine/threonine protein kinases that mediate intracellular signal transduction in response to various stimuli. Activation of the pathways rapidly alters the pattern of gene expression. To date, three major MAPKs have been identified: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and p38 MAPK. All of

these kinases have been reported to be associated with the activation of apoptosis (5). Increasing evidence indicates that in addition to directing the phosphorylation of upstream transcription factors and co-activators which control immediate genes, MAPK cascades also act directly on chromatin proteins such as histone H3 to modify chromatin concomitant with gene induction (6,7). However, the involvement of MAPKs-mediated histone H3 acetylation in apoptosis is unclear.

Epigenetic changes including histone acetylation, histone methylation and DNA methylation are now thought to play important roles in the onset and progression of cancer in numerous tumor types (8). Dietary components selectively activate or inactivate gene expression by epigenetic regulation has been implicated as chemoprevention agent or developed for the treatment of cancer. HDAC inhibitors are being explored as cancer therapeutic compounds because of their ability to alter several cellular functions known to be deregulated in cancer cells (9). Recent investigators suggest that dietary components, including diallyl disulfide and sulforaphane, possessing the ability to inhibit HDAC enzyme have been associated with cancer prevention (9). Quercetin (3,3',4',5,7-petahydroxyflavone), which is found in fruits, vegetables, herbs and red wine, has been reported to exhibit antioxidative, anti-carcinogenic and anti-inflammatory effects (10-12). The molecular mechanisms behind the effects are largely unknown. It has been reported that quercetin strongly inhibits neoplastic cell transformation and inhibits the enzymes involved in cancer cell proliferation and cell signal transduction pathways including protein kinase C, tyrosine kinase, and DNA topoisomerase II. Quercetin mediates apoptosis by induction of stress proteins including heat shock proteins, disruption of microtubules and mitochondrial release of cytochrome *c*. Quercetin also augments TRAIL-induced apoptotic death involving the ERK signal transduction pathway (13). Although MAPK activation during quercetin treatment has been studied in a variety of cell types, the presence of epigenetic regulation is not clear. In this study, we observed that quercetin induced apoptosis in HL-60 cells by enhancing the expression of FasL, in part, through promotion of histone H3 acetylation.

Materials and methods

Cell culture. The HL-60 human promyelocytic leukemia cell was obtained from ATCC and maintained in a logarithmic growth phase in RPMI-1640 supplemented with sodium pyruvate, 10% fetal bovine serum (FBS; Invitrogen-Gibco), 1% penicillin/streptomycin and 1% non-essential amino acids. Cell lines were maintained at 37°C in an incubator with 5% CO_2 and 95% air.

Antibodies and reagents. Anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-phospho-JNK/SAPK (Thr^{183}/Tyr^{185}) and anti-phospho-p38 (Thr^{180}/Tyr^{182}) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERK1/2, JNK/SAPK, p38, caspase-3, -8, -9, PARP, Fas, FasL, Bid, t-Bid, cytochrome *c*, Tom20, c-fos, c-jun, C23, tubulin and ß-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated H3 and anti-acetylated H4 were obtained from Upstate Biotechnology (Lake Placid,

NY). The MEK/ERK inhibitor (PD98059), and JNK inhibitor (SP600125), were obtained from Calbiochem (La Jolla, CA). Other chemicals such as quercetin were purchased from Sigma-Aldrich (St. Louis, MO).

DNA fragmentation assay. The cells were rinsed with ice-cold PBS and harvested by pipetting. The cell pellets were resuspended and incubated in 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS and 0.1 μg/ml proteinase K at 60°C overnight. The digested cells were extracted for DNA with phenol/chloroform (1:1) and chloroform/isoamyl alcohol (1:24). The extracted DNA was precipitated and digested in 10 mM Tris-HCl (pH 5.0) containing 1 mM EDTA and 10 μg/RNase for 1 h at 37°C. DNA (10 μg) per sample was resolved by electrophoresis in a 1.8% agarose gel impregnated with ethidium bromide (0.5 μg/ml), and the DNA pattern was examined by ultraviolet transillumination.

Preparation of total cell extracts and immunoblot analysis. Cells were washed with PBS plus zinc ion (1 mM) and lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-buffer, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin and 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4°C, the mixtures were centrifuged (10000 x g) for 10 min, and the supernatants were collected as whole-cell extracts. The protein extracts were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The samples containing 50-100 μg of proteins were boiled in Laemmli sample buffer, separated on SDS polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) and blotted with the indicated primary antibodies. Proteins were visualized with horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratory, Inc., South San Francisco, CA) followed by chemiluminescence detection.

Preparation of mitochondria and cytosolic fractions. Mito-chondrial fraction of the cells was isolated using a mito-chondria isolation kit obtained from Pierce as per manu-facturer's instruction. Cells ($2x10^7$) were pelleted by centrifu-gation at 850 g for 2 min and were resuspended in 800 μl of reagent A in a microcentrifuge tube. Then, cells were incu-bated in ice for 2 min and subsequently homogenized in a precooled Dounce tissue grinder applying 40-50 strokes. Reagent C (800 μl) was added to the homogenized solution and thoroughly mixed by repeated inversion. The entire mixed solution was centrifuged at 700 g for 10 min, and the pellet was discarded. The supernatant was further centrifuged at 12000 g for 15 min, and the pellet was considered as intact mitochondria. This fraction was further lysed in lysis buffer and subjected to Western blot analysis.

Immunofluorescence staining. After treatment of cells with quercetin, cells were harvested, washed with PBS and fixed with 3.7% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min and non-specific binding was blocked by incubation with 2% BSA in PBS for 30 min. Cells were then incubated for 30 min at room temperature with the primary

antibody: mouse monoclonal anti-Bax (conformation specific clone 6A7, Sigma-Aldrich). Excess antibody was removed by washing the cover slips three times with PBS-2% BSA. Cells were then incubated with the mouse-conjugate FITC secondary antibody for 30 min at 37˚C. After washing three times with PBS-2% BSA, cover slips were mounted and then viewed under a fluorescence microscope.

DAPI staining. After treatment of cells with quercetin, cells were pipetted and collected and then washed once with ice-cold PBS. Cells were attached to the slide by cytospin (500 rpm, 5 min). They were then air-dried, fixed and stained with the DAPI (10 μg/ml). The stained cells were examined by fluorescence microscopy (magnification x400).

Reverse transcriptase-PCR. Total cellular RNA was extracted from cells using the TRI reagent method (Molecular Research Center). For the reverse-transcription reaction, 10 μl of reaction mixture (1 μg of total RNA, 1 μl of random decamers, 1 μl of 10X RT buffer, 2 μl of deoxynucleotide triphosphate mix, 0.5 μl of RNase inhibitor, 0.5 μl of reverse transcriptase and 4 μl of nuclease-free water) was prepared for each sample. The mixtures were incubated at 44˚C for 1 h and then at 92˚C for 10 min to inactivate the reverse transcriptase. The first-strand cDNAs were synthesized with a RETROscript kit (Ambion) and served as templates for the PCR. A high-fidelity PCR master kit from Roche was used to perform PCR. The following primers were used: human Fas sense 5'-CACTCG CAACCTCTCTCCC-3' and antisense 5'-AGAGTGTGTGC ACAAGGCTG-3'; human FasL sense 5'-TCAATGAAACTG GGCTGTACTTT-3' and antisense 5'-AGAGTTCCTCAT GTAGACCTTGT-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5'-ACCACAGTCCATG CCATCAC-3' and antisense 5'-TCCACCACCCTGTTGC TGTA-3'. PCR products were resolved by electrophoresis on a 1.8% agarose gel with 0.5 μg/ml ethidium bromide and photographed using an AlphaEase FC imaging system (Alpha Innotech).

Nuclear extract preparation. HL-60 cells were lysed by adding 25 μl NP-40 10% and gently passed through a 27-gauge needle. The nuclei were collected by centrifugation at 600 x g for 5 min and resuspended in 50 μl of 20 mM HEPES, pH 7.9, containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin and 10 μg/ml aprotinin. The tubes were placed at 4˚C on a rotator shaker for 30 min and centrifuged at 12000 x g for 5 min at 4˚C. The supernatants were used as nuclear extracts and frozen at 70˚C until used.

Histone deacetylase and histone acetyltransferase assay. Assays were performed using the colorimetric HDAC and HAT activity assay from BioVision (BioVision Research Products, Mountain View, CA, USA) according to manufacturer instructions. Briefly, for HDAC activity, 50 μg of nuclear extracts were diluted in 85 μl of ddH$_2$O; then, 10 μl of 10X HDAC assay buffer was added followed by the addition of 5 μl of the colorimetric substrate; samples were incubated at 37˚C for 1 h. Subsequently, the reaction was stopped by adding 10 μl of lysine developer and left for an additional
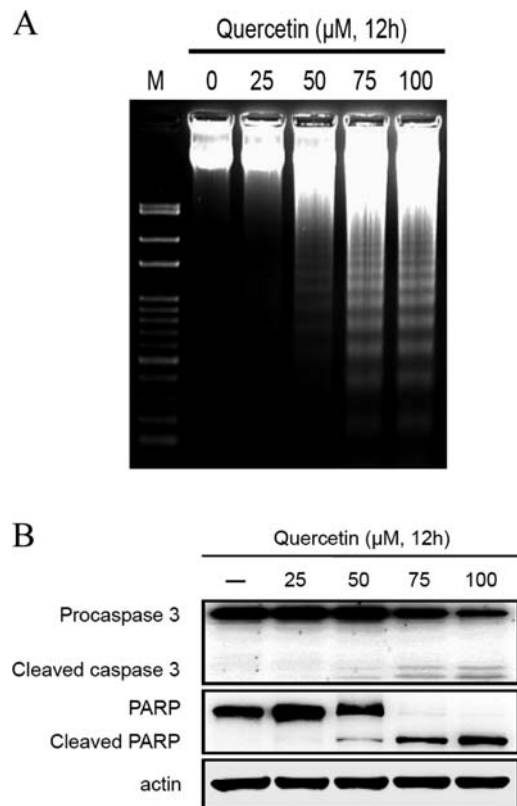

Figure 1. Quercetin-induced apoptosis in HL-60 cells. (A) Agarose gel electrophoresis of DNA from quercetin-treated HL-60 cells. HL-60 cells were treated with the indicated concentrations of quercetin for 12 h and genomic DNA was extracted, separated on an agarose gel and visualized under UV light by ethidium bromide staining. (B) Cells were incubated with 100 μM of quercetin for 12 h and cell lysates were assayed by Western blotting for the cleavage of procaspase-3 and PARP in HL-60 cells. ß-actin was used as a loading control. Data are a representative of the two independent experiments.

30 min at 37˚C. Samples were then read in an ELISA plate reader at 405 nm. For HAT activity, the adjusted weight of nucleic extracts (50 μg) was mixed with HAT substrate, followed by mixture with an enzyme mix. The samples were incubated at 37˚C for 1 h. Samples were then read in an ELISA plate reader at 440 nm.

Chromatin immunoprecipitation (CHIP) assays. CHIP assay was performed according to the manufacturer's instructions (Upstate). Briefly, HL-60 cells (1x10^6) with or without pre-treatment with PD98059 or SP600125 were administrated with quercetin (100 μM). Then cells were fixed with 37% formaldehyde, sonicated and immunoprecipitated with anti-Ac-H3. DNA isolated from the immunoprecipitated sample was amplified by PCR using FasL primers, 5'-CTGTAAATT ATGGTGATCGG-3' (forward), 5'-AACTCTAACAAAAT TGTTGTTCAG-3' (reverse), flanking the AP-1 consensus sequence. The PCR was carried out as follows: an initial denaturation at 94˚C for 3 min, 36 cycles of 94˚C 30 sec, 55˚C 30 sec and 72˚C for 10 min. The PCR product of FasL promoter was 206 bp.

Data analysis. Statistical significances were analyzed by one-way analysis of variance (ANOVA) with the post hoc Dunnett's test. P-values <0.05 were considered statistically
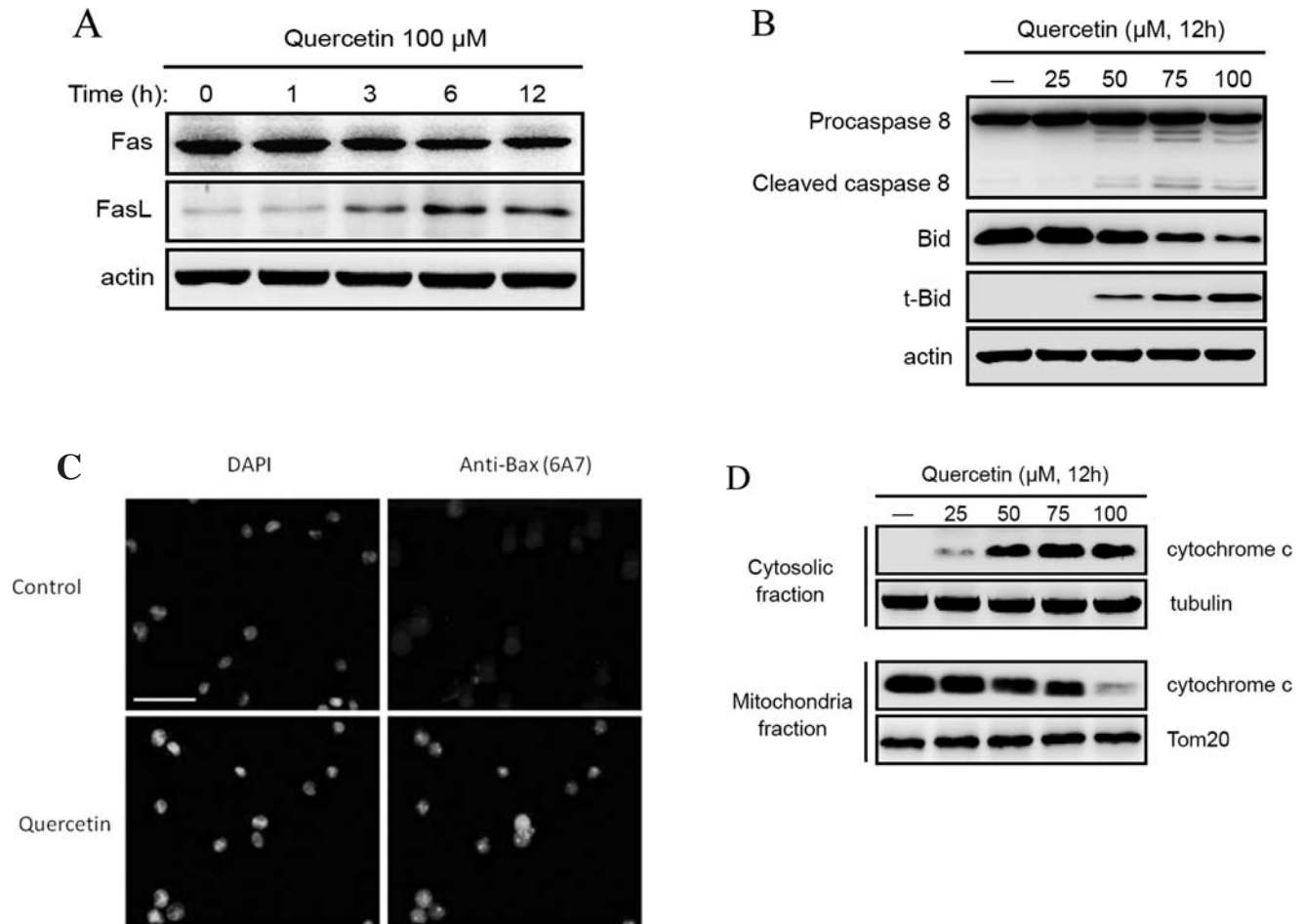
Figure 2. Effect of quercetin on the protein of FasL, caspase 8, t-Bid, Bax and cytochrome *c* in HL-60. (A) HL-60 cells were treated with 100 μM of quercetin for indicated time, and then total cell extracts were harvested. Western blot analysis was carried out with anti-Fas and anti-FasL antibodies, and anti-ß-actin was used as a loading control. (B) Western blot analysis was carried out with anti-caspase-8, anti-Bid and anti-t-Bid antibodies. ß-actin was used as a loading control. (C) HL-60 cells were treated with 100 μM of quercetin for 6 h and then immunostained with anti-Bax (6A7) to detect conformation-changed Bax. In addition, nuclear of HL-60 cells was stained with DAPI. Scale bar = 50 μm. (D) Cytosolic release of cytochrome *c* was measured by Western blot analysis in cytosolic and mitochondrial fraction. The tubulin and the Tom 20 were used as a cytoplasmic and a mitochondrial loading control, respectively.

significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA).

Results

Apoptotic induction by quercetin in HL-60 cells. In our preliminary study, quercetin induced cell death of HL-60 cells in a dose and time-dependent manner (data not shown). We further clarified the type of cell death. The HL-60 cells were treated with 0-100 μM quercetin for 12 h. As shown in Fig. 1A, after treatment with quercetin, the DNA showed the typical fragmentation patterns formed by inter-nucleosomal hydrolysis of chromatin. This demonstrates that quercetin exhibited apoptosis-inducing effects in HL-60 cells. In order to ascertain the effects of quercetin-induced apoptosis on the key aspect of apoptotic initiation via activation of caspase cascade in HL-60 cells, we performed a Western blot analysis to evaluate executioner caspases-3 and PARP, an intracellular biosubstrate of caspase-3. It was clearly shown that quercetin induced activation of caspase-3 and cleavage of PARP, both of which constitute hallmarks for apoptosis (Fig. 1B).

Activation of extrinsic apoptosis pathway by quercetin in HL-60 cells. The extrinsic apoptosis pathway involves binding of a ligand to one of the tumor necrosis factor families of death receptors, followed by activation of caspase-8 and caspase-3. Moreover, the intrinsic and extrinsic pathways are linked through the ability of caspase-8 to cleave Bid, which in turn leads to the conformation change of Bax in mitochondrial membrane and the release of cytochrome *c* from the mitochondria (3). We questioned whether the death receptor Fas/FasL system was implicated in the apoptosis of HL-60 cells by quercetin. As shown in Fig. 2A, treating cells with quercetin induced the increase of the expression of FasL protein after 3 h. By contrast, the expression of Fas protein was not modified by the treatment of quercetin. In addition, quercetin activated caspase-8 and cleaved Bid protein to its truncated form, t-Bid (Fig. 2B). Furthermore, we examined the effect of quercetin on conformation-changed Bax in mitochondrial membrane and cytochrome *c* release from mitochondria. By immunofluorescence staining with anti-Bax (clone 6A7), quercetin exhibited the induction of conformation-changed Bax (Fig. 2C). In addition, quercetin
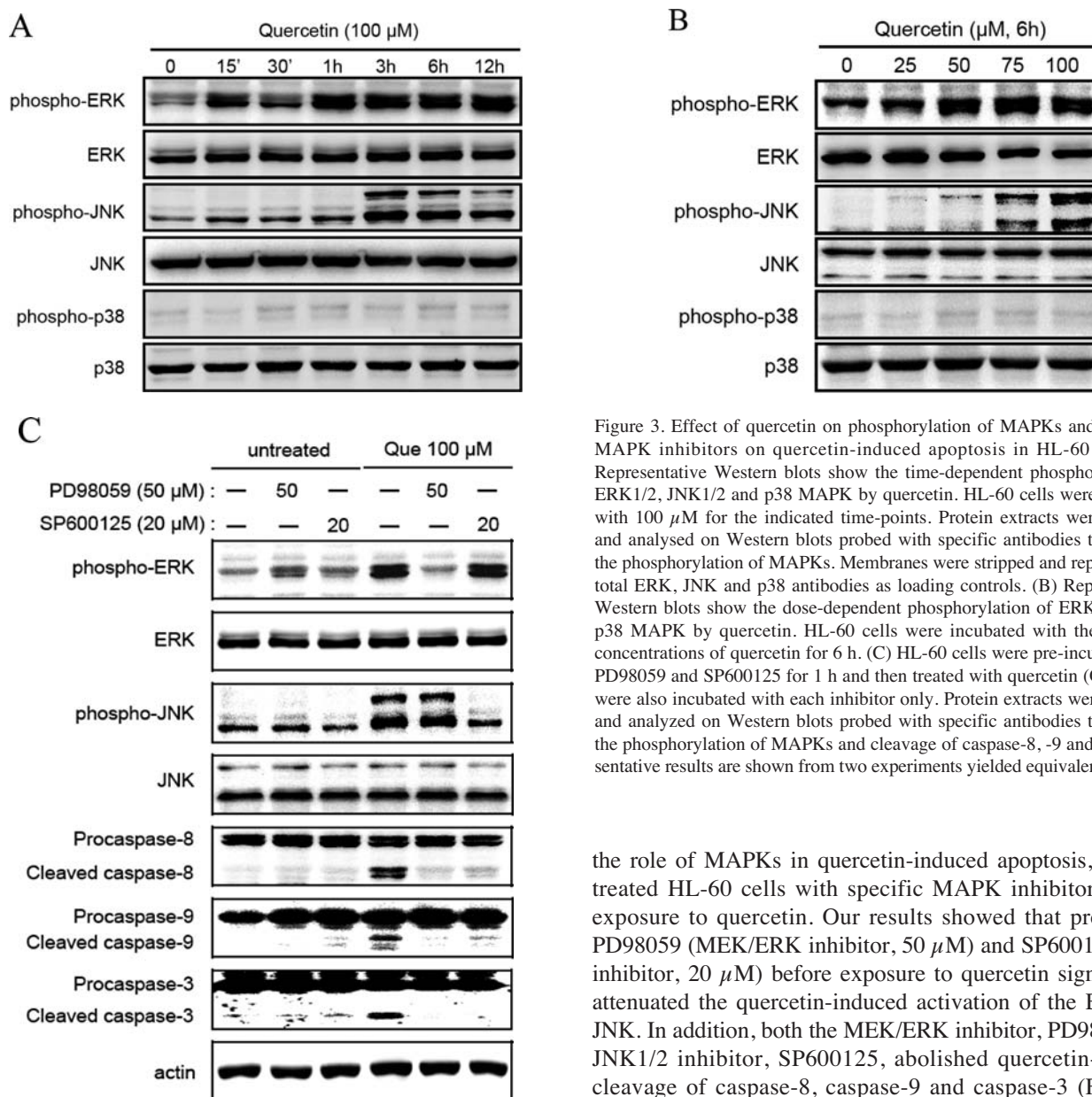
A Quercetin (100 µM)

0 15' 30' 1h 3h 6h 12h

phospho-ERK

ERK

phospho-JNK

JNK

phospho-p38

p38

B Quercetin (µM, 6h)

0 25 50 75 100

phospho-ERK

ERK

phospho-JNK

JNK

phospho-p38

p38

C

	untreated			Que 100 µM		
PD98059 (50 µM) :	—	50	—	—	50	—
SP600125 (20 µM) :	—	—	20	—	—	20

phospho-ERK

ERK

phospho-JNK

JNK

Procaspase-8
Cleaved caspase-8

Procaspase-9
Cleaved caspase-9

Procaspase-3
Cleaved caspase-3

actin

Figure 3. Effect of quercetin on phosphorylation of MAPKs and impact of MAPK inhibitors on quercetin-induced apoptosis in HL-60 cells. (A) Representative Western blots show the time-dependent phosphorylation of ERK1/2, JNK1/2 and p38 MAPK by quercetin. HL-60 cells were incubated with 100 µM for the indicated time-points. Protein extracts were prepared and analysed on Western blots probed with specific antibodies to ascertain the phosphorylation of MAPKs. Membranes were stripped and reprobed with total ERK, JNK and p38 antibodies as loading controls. (B) Representative Western blots show the dose-dependent phosphorylation of ERK, JNK and p38 MAPK by quercetin. HL-60 cells were incubated with the indicated concentrations of quercetin for 6 h. (C) HL-60 cells were pre-incubated with PD98059 and SP600125 for 1 h and then treated with quercetin (Que). Cells were also incubated with each inhibitor only. Protein extracts were prepared and analyzed on Western blots probed with specific antibodies to ascertain the phosphorylation of MAPKs and cleavage of caspase-8, -9 and -3. Representative results are shown from two experiments yielded equivalent findings.

increased the release of cytochrome *c* from mitochondria (Fig. 2D). These results demonstrate that quercetin induced activation of extrinsic apoptosis cascade in HL-60 cells.

Involvement of ERK and JNK in quercetin-induced apoptosis in HL-60 cells. MAPK family proteins have been implicated in the proliferation, differentiation and death of cells (7). They are, in general, subdivided into three different pathways, namely the ERK, p38 kinase and JNK signaling pathways (14). In view of evidence that ERK, JNK/SAPK and p38 MAPK play a critical role in cell fate, the effects of quercetin on the activation of MAPKs were examined. We treated HL-60 cells with quercetin, then used Western blotting to examine the phosphorylation of ERK, JNK and p38 MAPK. Our results showed that exposure to quercetin increased both ERK and JNK phosphorylation (Fig. 3A and B). However, p38 MAPK was not activated by treatment with quercetin in HL-60 cells. To further confirm these results and to examine

the role of MAPKs in quercetin-induced apoptosis, we pre-treated HL-60 cells with specific MAPK inhibitors before exposure to quercetin. Our results showed that pretreating PD98059 (MEK/ERK inhibitor, 50 µM) and SP600125 (JNK inhibitor, 20 µM) before exposure to quercetin significantly attenuated the quercetin-induced activation of the ERK and JNK. In addition, both the MEK/ERK inhibitor, PD98059 and JNK1/2 inhibitor, SP600125, abolished quercetin-induced cleavage of caspase-8, caspase-9 and caspase-3 (Fig. 3C). These results indicate that the ERK and JNK pathways were involved in quercetin-induced apoptosis.

Induction of FasL mRNA and c-Jun activation by quercetin involving ERK and JNK pathways. As shown in Fig. 2A, the Fas/FasL signaling pathway plays a crucial role in the quercetin-induced apoptosis in HL-60 cells. However, treatment with 100 µM of quercetin did not modify the expression of the Fas receptor while it increased that of FasL. Thus, this study attempted to determine whether quercetin-induced FasL protein expression results from up-regulation of FasL mRNA by transcriptional regulation. Incubation of HL-60 cells with 50 and 100 µM of quercetin for 3 and 6 h resulted in induction of FasL mRNA in a dose- and time-dependent manner. By contrast, expression of Fas mRNA was constitutive (Fig. 4A). These results show that quercetin induces FasL expression via transcriptional activation of the FasL gene in HL-60 cells.

AP-1 is a collection of dimers composed of the Jun, Fos or ATF families of bZIP (basic region-leucine zipper) DNA binding proteins. During transcription activation, these
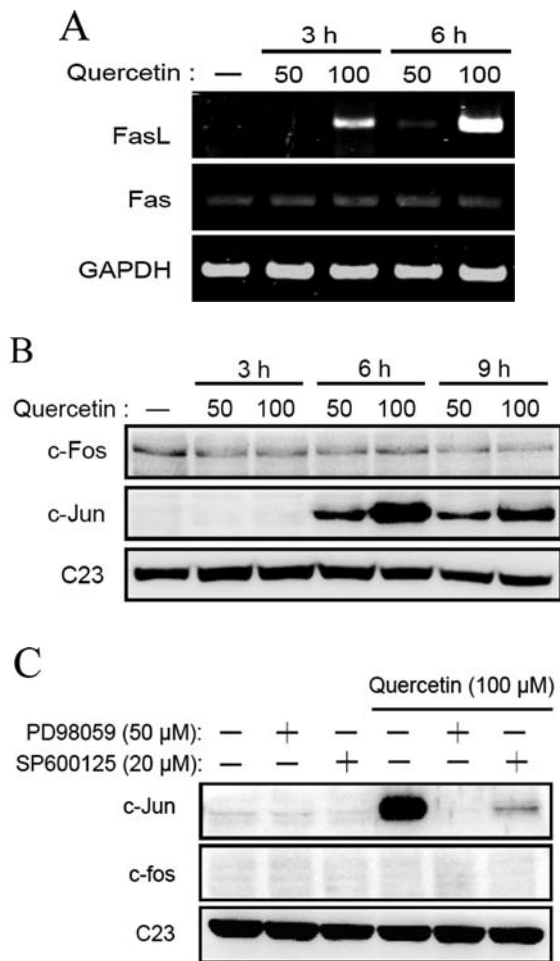
Figure 4. Effect of quercetin on nuclear c-Jun. (A) HL-60 cells were treated with 50 and 100 μM of quercetin, and total cellular RNA was extracted at the indicated times. Expression of Fas, FasL and GAPDH mRNAs was determined by quantitative RT-PCR using specific primers. (B) Time course and dose-dependence of quercetin effect on the translocation of c-Fos and c-Jun to nuclear. Cells were treated with 50 and 100 μM of quercetin for the indicated times and (C) cells were treated with 100 μM quercetin, 50 μM of PD98059 and 20 μM of SP600125 alone or in combination for 6 h. Then, cells were harvested by centrifugation at 600 x g for 10 min at 4˚C, and the nuclear fractions were prepared as described in the section of Materials and methods. To determine c-Fos and c-Jun translocation, the resulting nuclear fractions were analyzed by immunoblotting with antibodies specific for c-Fos, c-Jun and C23 as nuclear protein control. Representative results are shown from three experiments yielding equivalent findings.
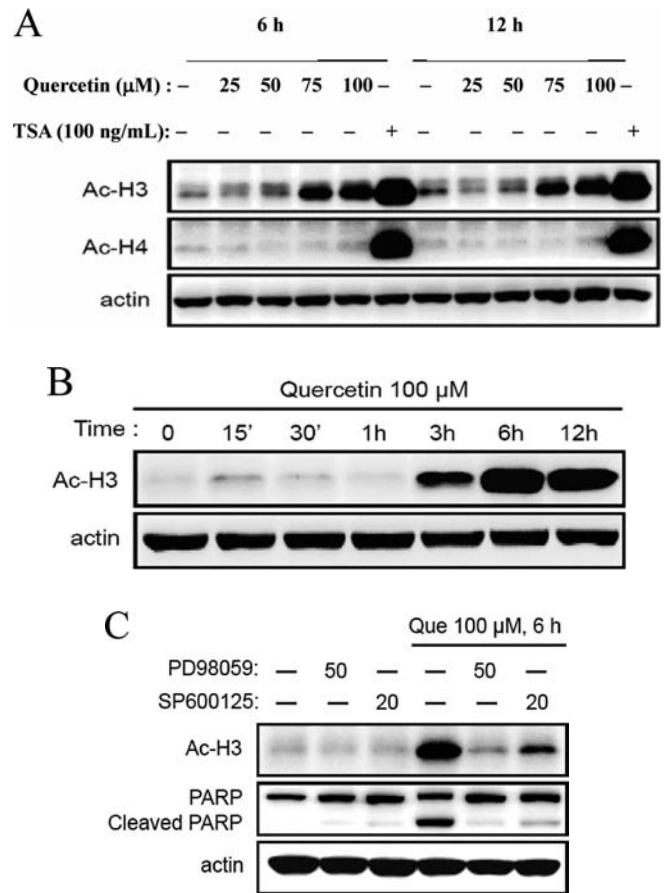

Figure 5. Quercetin-induced apoptosis involving increase acetylation of histone H3 through ERK and JNK pathways in HL-60 cells. (A) Cells were treated with indicated concentrations of quercetin and 100 ng/ml of TSA for 6 and 12 h and the expression of Ac-H3 and the acetylation of H4 (Ac-H4) were assessed. (B) By immunoblotting analysis, time course effect of quercetin (100 μM) on the acetylation of histone H3 (Ac-H3) was assessed. (C) Cells were treated with 100 μM of quercetin (Que), 50 μM of PD98059 and 20 μM of SP600125 alone or in combination for 6 h. The expression of Ac-H3 and cleaved PARP were analyzed by immunoblotting analysis.

dimers bind to a common *cis* acting element known as the AP-1 site in nuclear. Previously, it has been shown that activation of AP-1 mediating FasL expression triggers cell apoptosis (15). We therefore determined the effect of quercetin on the nuclear accumulation of c-Jun and c-Fos proteins. Cells were cultured in the presence or absence of 50 and 100 μM of quercetin for 3- 9 h, and nuclear protein was extracted. Nuclear extracts were separated by SDS-PAGE, and a Western blot analysis was performed for either c-Jun or c-Fos. Quercetin did not increase the accumulation of c-Fos protein; however, c-Jun was significantly increased after 6 h of quercetin exposure (Fig. 4B). We next determined whether quercetin-induced ERK and JNK phosphorylation was involved in c-Jun translocation. HL-60 cells were pretreated

with either PD98059 (MEK inhibitor) or SP600125 (JNK inhibitor) for 1 h, then cells were stimulated with 100 μM of quercetin for 6 h, nuclear protein was extracted, and a Western blot analysis was performed for c-Jun. Indeed, quercetin increased the nuclear c-Jun, whereas, there were significant decreases in the nuclear c-Jun in cells pretreated with PD98059 and SP600125 (Fig. 4C). Taken together, these data confirm previous findings which indicate that both the ERK and JNK MAP kinases are linked to AP-1 activation (16,17).

Effects of ERK and JNK pathways on quercetin-induced histone H3 acetylation. In Fig. 4A, it is interesting to observe that treatment with 100 μM quercetin induced FasL mRNA at 3 h, however, induction of c-Jun translocation required approximately 6 h. The discrepancy between FasL mRNA expression and c-Jun translocation suggests that other gene regulation programs may be required to activate the FasL gene. Previously, it has been shown that the acetylation and deacetylation of histones alter chromatin structure, which is suggested to play an important role in transcriptional regu-
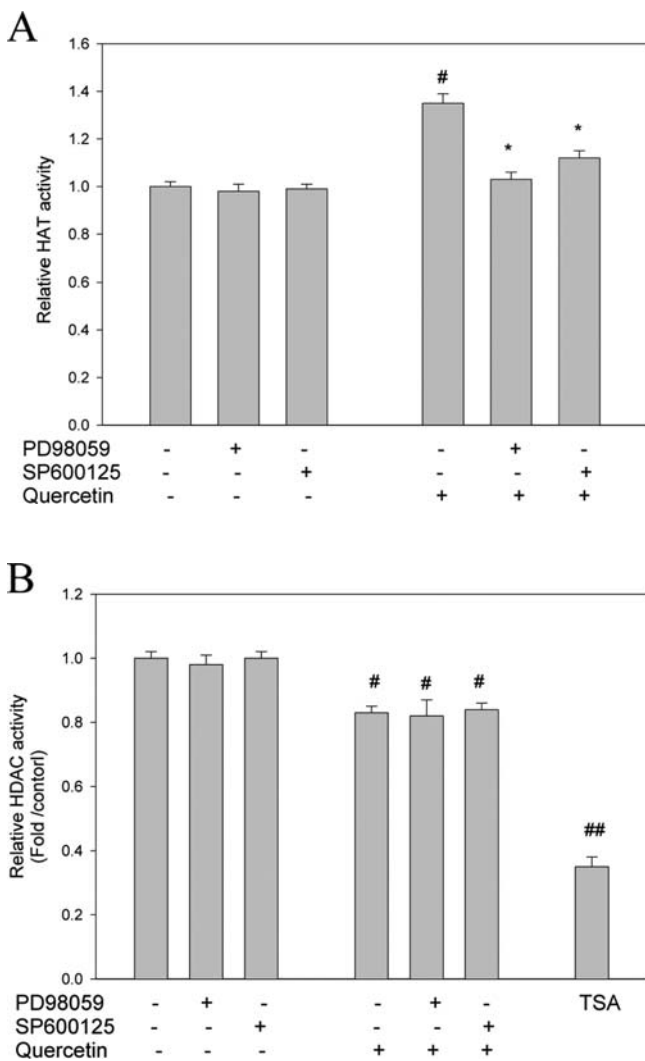
Figure 6. Association of ERK- and JNK-pathways with HDAC activity and HAT activity by treatment of quercetin. Cells were treated with 100 μM of quercetin, 50 μM PD98059 and 20 μM SP600125 alone or in combination for 6 h. (A) HAT and (B) HDAC activity were measured as described in Materials and methods.
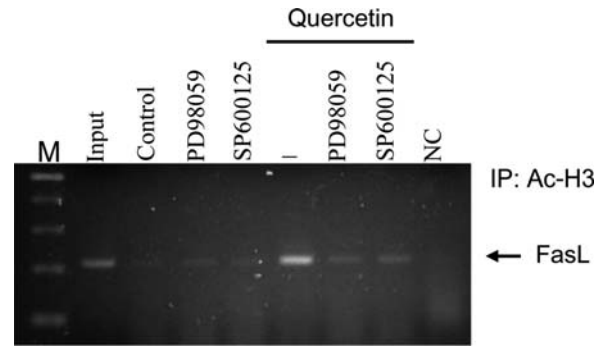

Figure 7. Involvement of hyperacetylation of histone H3 in quercetin-induced FasL expression in HL-60 cells. Soluble precleared chromatin was obtained from HL-60 cells pretreated with or without 50 μM of PD98059 (MEK inhibitor) and 20 μM of SP600125 (JNK inhibitor) for 2 h. Then, cells were treated with or without 100 μM of quercetin for 6 h. ChIP analysis of histone H3 acetylation (Ac-H3) at the loci was performed by immunoprecipitation (IP) with an antibody against Ac-H3. The FasL promoter sequences were detected by PCR with specific primers. To control input DNA, FasL promoter was amplified from initial preparations of soluble chromatin (before immunoprecipitation). PCR products obtained at 36 cycles are shown. A sample without the addition of DNA was used as negative control (NC).

acetylation in the 100 μM quercetin-treated cells. Likewise, treatment with JNK inhibitor (SP600125; 20 μM) reduced H3 acetylation (Fig. 5C). Consistent with these results, cleavage of PARP induced by quercetin was also almost completely abrogated by PD98059 and SP600125. These data indicate that quercetin-induced H3 acetylation and apoptosis are dependent, at least in part, on ERK1/2 and JNK signaling.

Effects of quercetin on HDAC and HAT. Because histone acetylation is regulated by a balance of opposing histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, we next determined whether quercetin affected the activity of HDAC and HAT and the role of ERK and JNK pathways in quercetin-induced modulation of HDAC and HAT. Nuclear extracts were prepared from treatment with different concentrations of quercetin in the absence or presence of MEK/ERK or JNK inhibitors, and then the total nuclear HAT and HDAC activities were analyzed. The results indicated that quercetin (100 μM) increased HAT activity (Fig. 6A) and decreased HDAC activity (Fig. 6B). The presence of MEK/ERK and JNK inhibitors did not significantly affect the quercetin-decreased HDAC activity (Fig. 6B) but significantly blocked quercetin-increased HAT activity (Fig. 6A). Taken together, these experiments suggest that the quercetin induced histone H3 acetylation through modulation of both HDAC and HAT activity, however, ERK and JNK signaling cascades are only involved in quercetin-induced HAT activation.

Involvement of Ac-H3 in quercetin-induced FasL up-regulation. To evaluate the promotion of the transactivation of FasL by histone H3 acetylation, CHIP assay was performed in HL-60 cells with or without treatment of quercetin. Using anti-Ac-H3 antibody followed by PCR with primers specific for the FasL promoter, the data showed that quercetin promoted histone H3 acetylation resulting in up-regulation of

lation (18). To evaluate whether quercetin altered the acetylation state of histone H3 and H4, HL-60 cells were administered with 25-100 μM of quercetin or 100 ng/ml of TSA, a histone deacetylase inhibitor, for the indicated time, and equal amounts of cell lysate protein were immunoblotted with specific antibodies for acetylated H3 and H4 or ß-actin. The data showed that quercetin (75 and 100 μM) increased histone H3 acetylation apparently, but did not affect histone H4 acetylation, while TSA increased both proteins (Fig. 5A). We further detected the time course effect (0-12 h) of quercetin on histone H3 acetylation. The results show that quercetin induced histone H3 acetylation with treatment for 3-12 h (Fig. 5B).

The MAPK cascade has been shown to increase histone acetylation in other systems (19,20). The question arises as to whether MAPK is involved in the increase of histone acetylation by quercetin. To examine this possibility, we used selective inhibitors of MAPK. Blockade of the ERK pathway with a MEK/ERK inhibitor (PD98059; 50 μM) decreased H3

FasL, whereas with pretreatments of PD98059 and SP600125 decreased the expression of FasL (Fig. 7). These results suggest that quercetin-induced up-regulation of FasL was associated with ERK and JNK-mediated epigenetic regulation.

Discussion

The relationship between diet and cancer has been implicated in several epidemiological studies. The results indicate that dietary phytochemicals have antineoplastic potential. Previous studies have demonstrated that quercetin induces apoptosis in a wide range of human cancer cells (12,13). A better understanding of the mechanisms by which quercetin induces apoptosis is necessary for its further development as a promising chemoprevention agent. In the present study, quercetin induced apoptosis and activated ERK and JNK signaling pathways in HL-60 cells (Figs. 1 and 3). Quercetin also increased expression of FasL in HL-60 cells, suggesting that the extrinsic apoptosis pathway is induced by quercetin (Fig. 2).

The Fas/FasL pathway has been implicated as an important cellular pathway regulating the induction of apoptosis in diverse cell types and tissue. ERK and JNK have been identified to contribute to death receptor transcription-dependent apoptotic signaling via c-Jun/AP-1, leading to transcriptional activation of FasL (21). In addition to c-Jun, the ERK and JNK signaling pathways have been found to activate other transcription factors as well as to alter histone acetylation, which contribute to AP-1 activity (5,6). Our data showed that quercetin-induced FasL mRNA expression occurred earlier than nuclear translocation of c-Jun (Fig. 4). This suggests that other gene regulation programs may be activated. CHIP assay showed that up-regulation of FasL by quercetin was associated with histone H3 acetylation (Fig. 7). Quercetin may mediate recruitment of chromatin remodeling complexes, coactivators and transcription factors to promote transcription of target genes.

Epigenetic modifications, such as histone acetylation and DNA methylation, are widely recognized as having a substantial role to play in both normal cellular physiology and disease processes, particularly in cancer where inappropriate gene-expression has long been known to play a fundamental role in the etiology of the disease (22). Increasing evidence suggests that induction of histone hyperacetylation is responsible for the antiproliferative activity and reversal of neoplastic characteristics through selective induction of genes (23). According to our results, quercetin exhibited the property of activating HAT mediated by the ERK and JNK signaling pathways. In addition, quercetin exhibited a weak inhibitory effect on HDAC activity which was independent of ERK and JNK signaling pathways. In Fig. 5, TSA, a histone deacetylation inhibitor, induced histone H3 and H4 acetylation while quercetin only induced histone H3 acetylation. Previously, it has been reported that histone deacetylation inhibitors (HDI) induce apoptosis through accumulation of excessive DNA damage in leukemia cells (24). Whether quercetin causes DNA damage in leukemia cells will be determined in the future. Histone acetylation data (Fig. 5), imply that there are different mediation in chromatin remodeling by quercetin and HDI. It is suggested that chemoprevention potential of dietary flavonoids may be mediated by epigenetic regulation. The association of flavonoid structure and potential of epigenetic regulation needs further investigation.

In conclusion, quercetin induced the expression of FasL through transactivation by the activation of ERK and JNK signaling pathway and the promotion of histone H3 acetylation in HL-60 cells. Thus, quercetin affected gene expression mediated leukemia apoptosis via targeting signaling pathway and chromatin remodeling. These results provide an important link to relevance for quercetin as a chemopreventive agent.

Acknowledgements

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