Suppression of Quercetin-Induced Autophagy Enhances Cytotoxicity through Elevating Apoptotic Cell Death in Human Bladder Cancer Cells

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Abstract

Objective: Quercetin, a natural dietary compound, has been demonstrated with antitumor activities against several types of cancers by disrupting cell cycle and inducing apoptotic cell death. However, human bladder cancer cells such as 5637 and T24 cells expressing mutant p53 are resistant to a 24 hrs quercetin treatment. In this study, the anticancer effect of quercetin was evaluated in these bladder cancer cells.

Materials and Methods: The bladder cancer cells treated with quercetin were subjected to evaluated cell apoptosis by caspase activity, TUNEL assay and cell viability assay. The cell autophagy was assessed by detecting procession of LC3-II autophagic marker protein.

Results: After 48 and 72 hrs of incubation, quercetin was found to be significantly effective in inhibiting proliferation of 5637 and T24 cells in a dose-dependent manner. Quercetin treatment increased the caspase 3/7 activities, percentage of subG0/G1 cells, and DNA fragmentation, indicating an induced apoptotic cell death. Pretreatment of a pan-caspase inhibitor, Z-VAD-FMK, attenuated the quercetin-decreased cell viability, suggesting that the cytotoxicity caused by quercetin mainly via apoptotic cell death. We also found that quercetin induced autophagy, as evidenced by the increased processing of LC3-II, a specific marker of autophagy. The disruption of autophagic flux by using bafilomycin A1, an autophagy inhibitor, caused significant accumulation of cellular p62 and LC3-II. In addition, the pretreatment of autophagy inhibitors, Baf A1 and chloroquine, strongly augmented apoptosis in 5637 and T24 cells, indicating the suppression of quercetin-induced autophagy enhanced apoptosis. Furthermore, the decreased cell viability and increased LC3-II processing were attenuated in quercetin-treated cells which pretreated with a reactive oxygen species (ROS) scavenger, N-acetyl cystine (NAC) suggested that quercetin-induced cytotoxicity and autophagy were initiated by the generation of ROS. Conclusion: This study proposes that combined treatment of autophagy inhibitor which sensitizes cells to quercetin treatment may be a better therapeutic approach to reduce bladder cancer cells proliferation.

Keywords: Apoptosis, autophagy, bladder cancer, quercetin, reactive oxygen species

INTRODUCTION

Transitional cell carcinoma of the bladder is a common urological cancer in developed countries.[1] There are two categories of
bladder cancer (BC): superficial (low-grade; nonmuscle-invasive BC [NMIBC]) and invasive (high grade; muscle-invasive BC [MIBC]). Most of the low-grade BC are treated by surgical removal with transurethral resection of bladder tumors (TURBT). However, due to its high recurrence rate, the use of intravesical agents such as mitomycin C and Bacillus Calmette–Guerin (BCG) is the standard procedure for treating superficial BC. During the past 2 decades, the 50% overall survival at 5 years has not improved because of the limited treatment options for BC. Therefore, novel therapeutic approaches are warranted other than the neoadjuvant platinum-based chemotherapy which did not significantly improve the overall survival of BC.

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone), a natural flavonoid molecule, is an antioxidative compound ubiquitously distributed in fruits, leaves, and grains in plants. It has anticancer effects which are linked to the capacity for targeting tumorigenic pathways by its antioxidative activity, inhibition of carcinogen-activating enzymes, modification of signal transduction pathways, and interactions with receptors and other proteins.[3] Quercetin has been demonstrated to enhance water transport in toad bladder. By suppressing the expression of P53 and Survivin proteins, quercetin inhibits cellular growth and induces apoptosis in BC cells.[5] It has been shown that quercetin increases the expression of Ca-activated K channel proteins to inhibit the growth of BC cells. More recently, quercetin was demonstrated to alter the extracellular catabolism of nucleotides, resulting in the accumulation of AMP.[6] Su et al. took one step further by showing quercetin induces apoptosis by activating S5'-AMP-activated protein kinase (AMPK) signaling in BC cells.

Macroautophagy (autophagy) is a highly conserved catabolic process which degrades damaged cellular organelles and misfolded protein to maintain cellular homeostasis when cells are under metabolic stress and nutrient deprivation.[7] The Novel Prize in Physiology or Medicine was awarded to Professor Yoshinori Ohsumi for his discoveries of autophagic mechanisms.[8] Accumulating studies have demonstrated that cancer cells induce autophagy against anticancer treatment by evading apoptotic pathways.[9] Therefore, targeting autophagy becomes a novel approach to enhance the efficacy of anticancer agent in various types of cancer. For example, our previous studies demonstrated that inhibition of basal autophagy or anticancer agents-induced autophagy enhanced the apoptotic cell death in BC cells.[10–12] It has been demonstrated that several signaling pathways involve in the regulation of autophagy. The mammalian target of rapamycin (mTOR) that governing the protein expression in cells is the major regulator to inhibit autophagy in a nutrition-rich condition or the presence of growth factors’ signaling.[13] In contrast, AMPK that responses to low energy or nutrient deprivation control autophagy induction.[14] Recently, Su et al., reported that quercetin-induced apoptosis in BC cells by activation of AMPK signaling.[15] Therefore, it is reasonable to hypothesize that quercetin may induce autophagy through AMPK activation. Here, we address the question of whether autophagy is significantly linked with quercetin-induced apoptosis in human BC cells. We reevaluated the induction of cell death by quercetin at various concentrations and incubation times and investigated the role of autophagy in quercetin-induced apoptosis in p53-mutant 5637 and T24 cells, with the aim of exploring the effective anticancer activity of quercetin. Our results demonstrate that quercetin induces reactive oxygen species (ROS)-mediated autophagy in these BC cells, and suppression of quercetin-induced autophagy further enhanced quercetin-induced apoptosis. Thus, inhibiting autophagy may be considered as a novel therapeutic approach which sensitizes cells to quercetin treatment to reduce BC proliferation.

Materials and Methods
Reagents
All the cell culture reagents including RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, and nonessential amino acids were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the chemicals including quercetin were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Primary antibodies against LC3, p-62, AMPK, p-AMPK (Thr172), mTOR, p-mTOR (Ser2448), p-mTOR (ser2481), and Beclin-1 were from Cell Signaling Technology (Danvers, MA, USA). Primary antibody against β-actin was from Sigma-Aldrich. The APO-DIRECT kit was acquired from BD Biosciences (San Jose, CA, USA). A Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific; and polyvinylidene fluoride (PVDF) membranes and ECL chemiluminescence kit were from Millipore (Bedford, MA, USA).

Cell culture
Human transitional cell carcinoma cell lines 5637 (ATCC#HTB-9) and T24 (ATCC#HTB-4) were obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and cultured in RPMI-1640 medium. These cells have performed STR-PCR profile at BCRC and were maintained at 37°C under 5% CO2, as described.[16] Media were supplemented with 10% FBS, 2 mM GlutaMAX-1, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with the indicated concentrations of quercetin while control cells received an equal volume of dimethyl sulfoxide (DMSO). The final concentration of DMSO was <1%.

Cell viability assays
The effect of quercetin on 5637 and T24 viability was determined by the WST-1 reagents as described.[17] Briefly, cells were seeded in 96-well plates for 16 h before the treatment of quercetin. The medium was removed, and 100 μl of complete medium with various concentrations of quercetin was added and incubated for 24, 48, and 72 h. After the treatment, the medium was refreshed with 100 μl of complete medium with WST-1 reagents per the manufacturer’s instruction. The cells were incubated at 37°C for another hour; then the plates were read immediately at 440 nm with a reference wavelength of 600 nm on an Epoch plate reader (Biotek Instruments, Waltham, MA, USA).
Assessment of apoptosis
The apoptosis induction in cells treated with quercetin was achieved by (a) the activities of caspase 3/7, (b) DNA fragmentation, and (c) the proteolytic cleavages of caspase 3 and poly (ADP-ribose) polymerase (PARP). For the detection of caspase 3/7 activity, a synthetic substrate (Z-DEVD) was used as described. Briefly, cells after treatment were lysed directly by adding caspase 3/7 assay buffer containing the substrate, and the lysates were incubated at 4 °C for 1 h. The fluorophore R110 released proteolytically from the substrate was then measured using a fluorescence plate reader (Victor X2, PerkinElmer, Inc., Waltham, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For the measurement of DNA fragmentation in quercetin-treated cells, a BD APO-DIRECT™ kit (Becton Dickinson, BD; NJ, USA) was employed per the manufacturer’s instructions. The level of DNA fragmentation was detected using an Accuri C5 flow cytometer, and the data were analyzed using the built-in software (BD). To detected the cleaved caspase-3 and PARP, total protein from the treated cells was subjected to the immunoblotting procedure as described. In some experiments, cells were pretreated with 200 nM Baf A1 for 2 h before the quercetin treatment.

Detection of autophagy
Autophagy induction in quercetin-treated cells was detected by the procession of LC3-II autophagic marker protein as described. In brief, cells subjected to the indicated treatments were harvested and lysed, and the protein concentration was determined by a BCA protein assay. An equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membrane was probed with antibodies against LC3, and the subsequent immunoblotting procedures were performed using a chemiluminescence process as per the manufacturer’s instructions. To detect the autophagic flux, the expression level of LC3-II was detected in a quercetin-treated cell with or without 2 h pretreatment of 200 nM Baf A1.

Reactive oxygen species generation
To detect the ROS generation in quercetin-treated cells, ROS indicator H$_2$DCFDA was utilized as described. Briefly, cells after the indicated treatments were stained with 1 μM H$_2$DCFDA, and the fluorescence was measured using the flow cytometer. In some experiments, the cells were pretreated with N-acetyl cysteine (NAC), Baf A1, or Z-VAD-FMK for 2 h before quercetin exposure and analysis of ROS generation.

Western blot
The protein levels in cells were examined using Western blot analysis as described for immunoblotting for LC3-II. The intensity of the immunoreactive bands was determined by densitometry scanning using ImageJ software (http://rsbweb.nih.gov/ij/). The results are expressed as the means ± standard deviation (SD) of three independent experiments.

Statistical analysis
The data are expressed as the means ± SD from triplicated experiments. The statistical evaluation was determined using a two-tailed Student’s t-test; and the differences were considered statistically significant at $P < 0.05$. All analyses were carried out using SigmaPlot Version 10.0 (Systat Software Inc., Chicago, IL, USA).

RESULTS
Quercetin induces apoptotic cell death in 5637 and T24 cells
The cell viability in cells upon quercetin treatment was accessed by WST-1 reagent to demonstrate the cytotoxic effects of quercetin. As shown in Figure 1, quercetin reduced 5637 and T24 cell viability in a dose- and time-dependent manner. A significant decreasing in cell viability was observed after 48 and 72 h of incubation at concentrations over 40 and 60 μM, respectively. The results indicated that quercetin led to an inhibition of cell proliferation, and IC$_{50}$ was shown in Table 1. The 5637 cells were more sensitive to quercetin at 72 h, with an IC50 of 47.91 μM after treatment. To access the quercetin-induced apoptosis, caspase 3/7 activity was detected in cells exposed to quercetin (0–120 μM) at 24, 48, and 72 h. In accordance with the decreased cell viability, the caspase 3/7 activity in quercetin-treated cells increased gradually according to the treatment doses and durations [Figure 2a]. In addition, the percentage of subG0/G1 cells and the TUNEL-positive cells were increased in 5637 and T24 cells treated with 60 or 120 μM quercetin [Figure 2b and c]. Furthermore, pretreatment of 1 mM Z-VAD-FMK attenuated quercetin-reduced cell viability in 5637 and T24 cells [Figure 2d], indicating quercetin induces apoptotic cell death in these BC cells.

Quercetin induces protective autophagy in 5637 and T24 cells
Our results showed that quercetin-induced cell apoptosis at 48 and 72 h posttreatment obviously. Previous studies have indicated that quercetin induces protective autophagy and apoptosis in various cancer cells. We hypothesize that quercetin may also exert dual roles in apoptosis and protective autophagy. To test this hypothesis, the autophagic marker protein, LC3-II, was detected in cells treated with 0–120 μM quercetin for 24 h. The result showed that quercetin-induced moderate apoptosis and might be caused

<table>
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<th>Cells</th>
<th>Inhibitory concentration 50% (IC50) of quercetin</th>
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<td></td>
<td>5637</td>
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<td></td>
<td>24</td>
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<tr>
<td>IC50 (μM)</td>
<td>47.91</td>
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by protective autophagy effect. As shown in Figure 3a, the processing of LC3-II was gradually increased on increasing the concentrations of quercetin, suggesting the formation of autophagosomes in the quercetin-treated 5637 and T24 cells. Furthermore, an autophagy inhibitor, Baf A1 which inhibits the fusion of autophagosomes to lysosomes, caused significant accumulation of cellular LC3-II in quercetin-treated cells [Figure 3b] in BC cells. These results indicate that quercetin induces autophagy in 5637 and T24 cells.

To understand the role of quercetin-induced autophagy in these BC cells, the cell viability in quercetin-treated cells with or without 2 h pretreatment of Baf A1 was detected. The suppression of quercetin-induced autophagy by Baf A1 resulted in the decreased cell viability, suggesting that quercetin-induced autophagy serves as a protective role in these BC cells on quercetin treatment [Figure 4].

**Suppression of quercetin-induced autophagy enhances apoptosis**

We next detected the level of apoptosis in quercetin-treated cells with or without the pretreatment of autophagy inhibitor, Baf A1. As shown in Figure 5a, cells with or without 200 nM Baf A1 pretreatment were treated with 60 or 120 μM quercetin for 24 h; then, the expression levels of cleaved PARP and cleaved caspase 3 (c-Casp3) were detected by Western blot. The results indicated that proapoptotic markers were increased by pretreatment with Baf A1. These finding revealed that inhibition of quercetin-induced autophagy promoted apoptosis and confirmed that quercetin-induced autophagy in BC cells. Furthermore, we detected the DNA fragmentation in quercetin-treated cells with or without autophagy inhibitors, Baf A1 or chloroquine (CQ), at 48 h posttreatment. As shown in Figure 5b, the levels of TUNEL-positive cells were significantly increased in quercetin-treated cells pretreated with both autophagy inhibitors. These results demonstrated that suppression of autophagy enhanced the apoptosis induction judged by the increased levels of pro-apoptotic proteins and DNA fragmentation. In summary, suppression of quercetin-induced autophagy significantly enhances the quercetin-induced cytotoxicity by elevating the apoptosis.

**Quercetin-induced autophagy is mediated by reactive oxygen species generation**

Quercetin has been reported to generate ROS and causes free radical-induced apoptosis through the AMPK/p38 and AMPK/COX2 pathway. Our previous study demonstrated...
that autophagy induction was mediated by ROS in BC cells.\cite{18}
It is possible that autophagy induction in quercetin-treated
cells was mediated through the generation of ROS. To test this
hypothesis, cell viability was detected at 48 h posttreatment in a
quercetin-treated cell with or without the pretreatment of a ROS
scavenger, NAC. As shown in Figure 6a, pretreatment of NAC
attenuated the loss of cell viability in quercetin-treated cells.
Moreover, NAC pretreatment also attenuated the processing
of LC3-II autophagic protein [Figure 6b], suggesting that
quercetin-induced autophagy is initiated, at least partially, by
ROS generation.

**DISCUSSION**

BC is one of the main malignancies in the genitourinary
tract.\cite{16,17} The major problem of BC is that even with the routine
surveillance, repeated TURBT and the use of intravesical
agents such as BCG, there is still a subset of patients who
progress from NMIBC to MIBC that drive the mortality of
this disease. When new treatment regimens are developed
rapidly to manage other types of cancer, the treatment option
for BC is still limited. For this reason, there are many research
groups which have studied the natural products generated
from the secondary metabolism of plants aiming to find novel
treatments against BC. Quercetin exists in nearly all plants has
been a part of the human diet for a long time.\cite{26} Accumulating
studies demonstrate that quercetin exerts biological and
pharmacological activities that are beneficial to human health.
Such characteristics including the selective antiproliferative
effect and cytotoxicity through the induction of apoptosis in
cancer cell lines. For example, quercetin has been reported to
inhibit the invasion, migration, and proliferation in prostate
cancer cells, but with minimal impact on normal prostate
epithelial cells.\cite{27} Quercetin is also reported to be effective
against the primary culture of papillary urothelial carcinoma
through inhibiting cell proliferation and colony formation
through inducing DNA damage.\cite{28}

In the present study, we found that cell proliferation
in quercetin-treated cells was significantly suppressed
at 48 and 72 h posttreatment. We also evaluated and
confirmed that quercetin-induced apoptosis in BC

**Figure 2:** Cytotoxicity induced by quercetin was through the induction of apoptotic cell death. (a) The caspase 3/7 activity was detected in 5637
and T24 cells treated with indicated concentrations of quercetin for 24, 48, and 72 h. (b) The percentage of sub G0/G1 cells was measured in cells
treated with 0, 60, and 120 μM quercetin (c) The level of DNA fragmentation was detected in cells treated with 0, 60, and 120 μM quercetin. (d)
The cell viability in 60 μM quercetin-treated cells with or without 2 h pretreatment of 1 mM Z-VAD-FMK, a pan-caspase inhibitor, was detected.
*P <0.05 compared to control; **P < 0.05 compared to quercetin-treated cells.
cells. The administration of Z-VAD-FMK attenuated quercetin-decreased cell viability suggested that apoptotic cell death is responsible for quercetin-induced cytotoxicity. Recently, the cytotoxicity study of quercetin and its derivative on BC cells have been investigated. The result showed that quercetin has a moderate cytotoxic effect on normal bladder cells.\[29\] The IC50 of quercetin against BC cells was reported to be ~60 μM. The higher dose (60 and 120 μM) of quercetin used in this study could help us to develop quercetin as an intravesical chemotherapy reagent with fewer systemic side effects.

In the current study, we demonstrate that quercetin induces protective autophagy in human BC cells. At 24 h posttreatment, the expression of LC3-II was increased dose-dependently in quercetin-treated cells. The inhibition of quercetin-induced autophagy using Baf A1 resulted in significant accumulation of LC3-II and p62 protein, indicating a disruption of autophagic flux in quercetin-treated cells. It has been reported that quercetin induces autophagy in colon, gastric, breast, cervical, ovarian, and B-lymphoblastoid cancer cells.\[22,30,31\] Similar to the Wang et al. study,\[22\] our results revealed that quercetin-induced apoptosis and autophagy in BC cells. Moreover, Baf A1 pretreatment further decreased the cell viability, suggesting a protective role of quercetin-induced autophagy. These results are in agreement with the previous finding of the Wei et al.,\[32\] suggesting that quercetin elicits protective autophagy in BC cells. The administration of autophagy inhibitors including Baf A1 or CQ enhanced the quercetin-induced apoptosis detected by the elevated levels of cleaved PARP, c-Casp3, and DNA fragmentation. The previous study has shown that Baf A1 could induce apoptosis in osteosarcoma cells.\[33\] In accordance with this report, our data showed that Baf A1 has a little effect on cell viability at a lower concentration. Moreover, pretreatment with Baf A1 decreased cell viability in BC cells, especially in T24 cells. This result may be caused by the high basal level of autophagy in T24 cell.\[17\] In cancer-associated fibroblast cells, quercetin treatment has been demonstrated to promote the removal of defective mitochondria which is induced by oxidative stress, resulting in the “reverse Warburg effect” whereby these fibroblasts provided nutrients to stimulate

Figure 3: Quercetin-induced autophagy in 5637 and T24 cells. (a) The procession of the autophagic marker protein, LC3-II, was increased dose-dependently on quercetin treatment. (b) Disruption of autophagic flux by autophagy inhibitor, bafilomycin A1. Data are present as the mean ± standard deviation (S.D); *P<0.05 compared to Baf A1 untreated group.
mitochondrial biogenesis and oxidative metabolism in adjacent cancer cells.\(^{34}\) Taken together, quercetin induces protective autophagy in different types of cancer, and our data strongly suggest that inhibiting quercetin-induced autophagy enhances apoptotic cell death in human BC cells.

Quercetin is ubiquitously present in foods including vegetables, fruit, tea, and wine as well as in many food supplements and considered to be one of the most prominent dietary antioxidants.\(^{35}\) However, our data suggested that quercetin-induced ROS generation was responsible for the induced cytotoxicity and autophagy in human BC cells. Consistent with our finding, quercetin has been reported to induce ROS production which involves in quercetin-induced apoptosis in human hepatoma cells.\(^{36}\) Quercetin is demonstrated to increase intracellular ROS levels by forming quercetin-radicals after peroxidase-catalyzed oxidation to scavenge reactive peroxyl radicals.\(^{37}\) On the
other hand, quercetin can alter ROS metabolism by inhibiting the intracellular pool of glutathione. In several models, quercetin-induced glutathione depletion resulted in apoptosis induction through mitochondria depolarization. The regulatory role for ROS of mitochondrial origin as signaling molecules in autophagy to either survival or cell death has been discussed previously. The moderate ROS level induces mitophagy to promote cell survival. However, accumulation of ROS may also trigger apoptosis pathway. Whether quercetin-induced ROS generation leads to mitochondria damages that are responsible for apoptosis and autophagy induction needs further investigation.

In conclusion, the results of this study show the antiproliferative potential of quercetin on BC cells by inducing apoptotic cell death. Utilizing the autophagy and ROS inhibitors, we showed that quercetin induces ROS-initiated autophagy which acts as a protective role in human BC cells. Since our previous study showed that a high basal level of autophagic activity was found in human BC cell lines, these results suggest that combined treatment of autophagy inhibitor which sensitizes cells to quercetin treatment may be a better therapeutic approach to reduce BC cells proliferation.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES
Tsai, et al.: Quercetin induces protective autophagy in BC cells


