View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Biochimica et Biophysica Acta 1823 (2012) 451–457

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Autophagy inhibition enhances ursolic acid-induced apoptosis in PC3 cells

Seoung Woo Shin, Sung Youl Kim, Jeen-Woo Park *

School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Republic of Korea

A R T I C L E I N F O

Article history:
Received 5 August 2011
Received in revised form 21 October 2011
Accepted 24 October 2011
Available online 8 December 2011

Keywords:
Prostate cancer
Ursolic acid
Autophagy
Apoptosis
Akt/mTOR

A B S T R A C T

The phosphoinositol 3-kinase/Akt pathway plays a critical role in oncogenesis and the dysregulation of this pathway through loss of PTEN is a particularly common phenomenon in aggressive prostate cancers. Several recent studies have indicated that ursolic acid (UA), a pentacyclic triterpenoid, and its derivatives inhibit the growth of cancer cells by cell cycle arrest and the stimulation of apoptosis. In the present study, we report a novel autophagic response of UA in PTEN-deficient PC3 prostate cancer cells. As one of the major types of programmed cell death, autophagy has been observed in response to several anticancer drugs and demonstrated to be responsible for cell death. UA-induced autophagy in PC3 cells is associated with the reduced cell viability and the enhanced expression of LC3-II, an autophagosome marker in mammals, and monodansylcadaverine incorporation into autolysosomes. Furthermore, we found that UA exhibited anti-proliferative effects characterized by G_1 phase arrest and autophagy at an early stage that precedes apoptosis. We also show that UA-induced autophagy in PC3 cells are mediated through the Beclin-1 and Akt/mTOR pathways. Inhibition of autophagy by either 3-methyladenine or Beclin-1/Atg5 small interfering RNA enhanced UA-induced apoptosis. Taken together, our data suggest that autophagy functions as a survival mechanism in PC3 cells against UA-induced apoptosis and a rational for the use of autophagy inhibitors in combination with UA as a novel modality of cancer therapy.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Prostate cancer is among the most commonly diagnosed male diseases and a leading cause of cancer mortality in men [1]. More than half of all men are diagnosed with cancer over the age of 70 years, with prostate cancer constituting about 50% of the cancers in this age group [2]. The development of prostate cancer in humans has been viewed as a multistage process involving the onset as small latent carcinoma of low histological grade to large metastatic lesion of higher grade [3]. Because chemotherapy and radiation therapy are largely ineffective, and metastatic diseases frequently develop even after potentially curative surgery, there are limited treatment options available for this disease [4–6]. In this context, novel ways of treating prostate cancer must be developed.

Ursolic acid (UA), a pentacyclic triterpenoid, has been isolated from many kinds of medicinal herbs and other plants such as *Eriobotrya japonica*, *Rosmarinns officinalis*, *Melaleuca leucadendron*, *Ocimum sanctum*, *Eugenia jambolana*, *Calluna vulgaris*, *Malus domestica*, *Vaccinium macrocarpon*, and *Glechoma hederaceae* [7]. Several recent studies have indicated that UA and its derivatives inhibit the growth of cancer cells through cell cycle arrest and the stimulation of apoptosis [8–10]. Recently, it has been shown that UA can potentiate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and sensitize resistant cancer cells to TRAIL [11]. In animal studies, UA has been shown to be chemopreventive [12] and to inhibit tumor invasion and metastasis of esophageal carcinoma [13,14]. Because UA is relatively non-toxic to normal cells [7], an important implication of these finding is that this agent might play a useful role in the treatment of cancer. However, little is known regarding the anti-tumor effects of UA as well as its underlying mechanisms on prostate cancer.

Autophagy, or type II programmed cell death, is morphologically characterized by a cell with an intact nucleus and an accumulation of cytoplasmic double-membraned autophagic vacuoles called autophagosomes[15,16]. These autophagosomes ultimately fuse with lysosomes to generate single-membraned autophagosomes capable of degrading their contents [17]. Autophagy is controlled by mammalian target of rapamycin (mTOR) downstream of phosphatidylinositol 3-kinase (PI3K)/Akt, which regulates cell growth and protein synthesis in response to nutrient and growth factor availability [18]. There is emerging evidence that autophagy plays an important role in the regulation of malignant cell survival [19].

In the present study, we report a novel autophagic response of UA in PC3 prostate cancer cells. The UA-induced autophagy in PC3 cells is associated with up-regulation and processing of autophagy-related protein microtubule-associated protein 1 light chain 3 (LC3) and its recruitment to the autophagosomes. Furthermore, we found that UA exhibited anti-proliferative effects characterized by G_1 phase arrest

* Corresponding author. Tel.: +82 53 950 6352; fax: +82 53 943 2762.
E-mail address: parkjw@knu.ac.kr (J.-W. Park).

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.10.014

and autophagy at an early stage that precedes apoptosis. The inhibition of UA-induced autophagy potentiates the anti-cancer effects of UA, which provides a novel strategy to enhance therapeutic efficacy.

2. Materials and methods

2.1. Materials

UA, 3-methyladenine (3-MA), acridine orange, monodansylcadaverine (MDC) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were obtained from Santa Cruz (Santa Cruz, CA) and Cell Signaling (Beverly, MA).

2.2. Cell culture

PC3 and DU145 cells were cultured in monolayers at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 using Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (50 U/ml), and 50 μg/ml streptomycin.

2.3. Cytotoxicity

To determine cytotoxicity of UA, cells (2×10^4) were grown until 80% confluence in 96-well plates, and cell viability after treatment with various concentrations of UA for 1–2 days was assessed by MTT assay. The absorbance at 540 nm with 620 nm reference was read with an ELISA plate reader. Viability was expressed as percentage of control.

2.4. Detection of acidic vesicular organelles

Formation of acidic vesicular organelles (AVOs), a morphological characteristic of autophagy, was detected by acridine orange staining [20]. Cells were stained with 1 μg/ml acridine orange for 15 min and samples were observed under a Zeiss Axiovert 200 inverted microscope (excitation, 546 nm; emission, 575/640 nm).

2.5. Visualization of autophagic vacuoles

The autofluorescent agent MDC was recently introduced as a specific autophagolysosome marker to analyze the autophagic process [21]. PC3 cells were treated with 40 μM UA for 24 h. Autophagic vacuoles were labeled with MDC by incubating cells with 50 μM MDC in PBS at 37 °C for 15 min. After incubation, cells were washed three times with PBS and immediately analyzed by a Zeiss Axiovert 200 inverted microscope (excitation, 390 nm; emission, 460 nm).

2.6. GFP–LC3 translocation

The green fluorescent protein (GFP)-fused LC3 was used to detect autophagy as previously described by [22]. PC3 cells were transfected with 2 μg of GFP–LC3 expression plasmid using Lipofectamine 2000. After 24 h, transfected cells were treated with UA for 24 h, and the distribution and fluorescence of GFP–LC3 were visualized by fluorescence microscopy.

2.7. Immunoblot analysis

Total protein extracts were separated on 10–12.5% SDS-PAGE and transferred to the nitrocellulose membrane. The membrane was incubated with the desired primary antibody, followed by a horseradish peroxidase-labeled anti-rabbit IgG. The immune-reactive bands were visualized by an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.8. DNA fragmentation

To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500 μl aliquot of the lysis buffer (100 mM Tris–HCl, pH 8.5, EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2×10^5 cells) and incubated at 37 °C overnight. DNA was obtained by ethanol precipitation, separated in a 0.8% agarose gel, and visualized under UV light.

2.9. Flow cytometric analysis

PC3 cells were collected at 2000×*g* for 5 min and washed twice with cold PBS, fixed in 70% ethanol for at least 2 h at −20 °C, decanted ethanol by centrifuge, and stained with 1 ml of PI staining solution (50 μg PI, 100 U RNase A, and 1.5% Triton X-100) for at least 1 h in the dark at 4 °C. The labeled cells were analyzed by flow cytometry. Apoptosis was quantified by measuring the sub-G_1 population on the cell cycle data acquired by flow cytometry.

2.10. RNA interference

siRNA sequences for Beclin-1 [23], Atg5 [24], and their scrambled controls were obtained from Invitrogen (Carlsbad, CA). Transfection of siRNA was performed with Lipofectamine 2000 according to the protocol provided by the manufacturer (Invitrogen).

2.11. Statistical analysis

All experiments were performed at least three times. The data were expressed as means ± S.D. The difference between two mean values was evaluated using the Student's *t*-test and was considered to be statistically significant when $p<0.05$.

3. Results and discussion

3.1. PC3 and DU145 have distinct PTEN expression and Akt activation

The PI3K/Akt pathway is the cell survival pathway that is important for normal cell growth and proliferation [25]. This pathway has also been implicated in tumorigenesis [26] and is becoming an important target for cancer therapy [27,28]. PI3K/Akt signaling is frequently activated in various types of cancers, and hence, represents a major cell survival pathway. Its activation has long been associated with malignant transformation and apoptotic resistance [29,30]. The tumor suppressor PTEN is a dual protein and phosphoinositide phosphatase that negatively controls the PI3K/Akt signaling pathway [31]. The loss of PTEN function is associated with a hyperactivated PI3k/Akt pathway [28,32]. To assess the role of PTEN status in response to UA treatment, *PTEN*−/− PC3 and *PTEN*+/+ DU145 prostate cancer cells were first screened by Western blot analysis to determine the PTEN expression and phosphorylation of Akt. As shown in Fig. 1A, PC3 cells demonstrated no PTEN expression, which correlated to high levels of phosphorylated Akt, whereas the DU145 cells displayed high PTEN levels, which correlated with no detectable levels of phosphorylated Akt. We next tested whether prostate cancer cells with variant PTEN status responded differently to the cytotoxic effects of UA. PC3 and DU145 cells were treated with increasing concentrations of UA. As shown in Fig. 1B, PC3 cells were more resistant to the antiproliferative effects of UA. PARP cleavage, an indicative of apoptosis, increased in DU145 cells compared to PC3 cells upon exposure to UA (Fig. 1C). These findings are in agreement with published reports indicating that Akt hyperactivation confers resistance to chemotherapy [32,33].

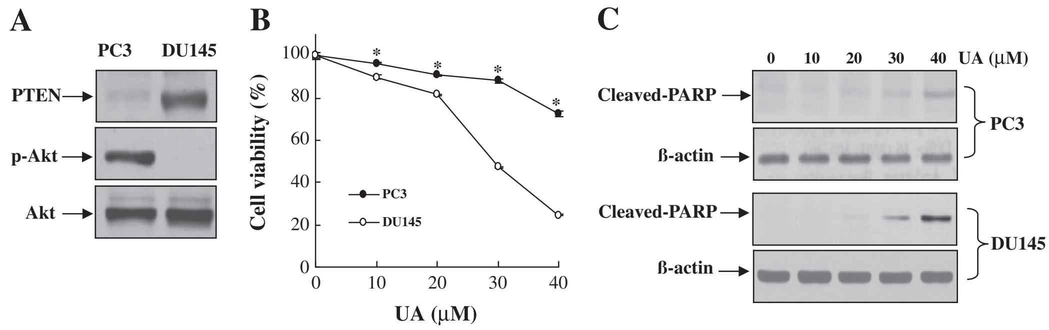

Fig. 1. PTEN status confers chemoresistance to UA in prostate cancer cells. (A) Twenty μg of protein lysate was separated on 10% SDS-PAGE by electrophoresis and immunoblotted for PTEN, phosphorylated Akt, and total Akt. (B) Treatment with increasing doses of UA (10–40 μM) for 24 h showed a higher cytotoxicity in the PTEN-positive DU-145 cells when compared with the PTEN-negative PC-3 cells. Cell viability was determined by MTT assay. Data are presented as means ± S.D. of three separate experiments. *$p<0.01$ versus DU145 cells exposed to UA. (C) Immunoblot analysis of the apoptotic marker protein expressed in prostate cancer cells. Cells were treated with 10–40 μM UA for 24 h. The cell-free extracts (20 μg protein) were subjected to 10% SDS-PAGE and immunoblotted with antibodies against cleaved-PARP. β-Actin was used as an internal control.

3.2. UA causes G_1 arrest

The in vitro anticancer activity is due to the combined induction of the apoptotic program, G_1 arrest, and reduced DNA synthesis [34]. To determine whether UA would induce cell cycle arrest, we analyzed the effect of UA on the cell cycle distribution using PI staining and flow cytometry. When PC3 cells were treated for 24 h with various concentrations of UA, the population of cells in the G_1 phase increased significantly from 43.13% to 63.35% at 40 μM of UA (Fig. 2A). Further examination of molecular markers associated with G_1 arrest showed remarkable changes, including increased p21 and p27 levels and reduced cyclin D1, D3 and CDK4 levels (Fig. 2B). The Western blot data are consistent with the G_1 arrest phenomenon observed in the flow cytometric analysis and these results suggest that UA markedly induces G_1 arrest.

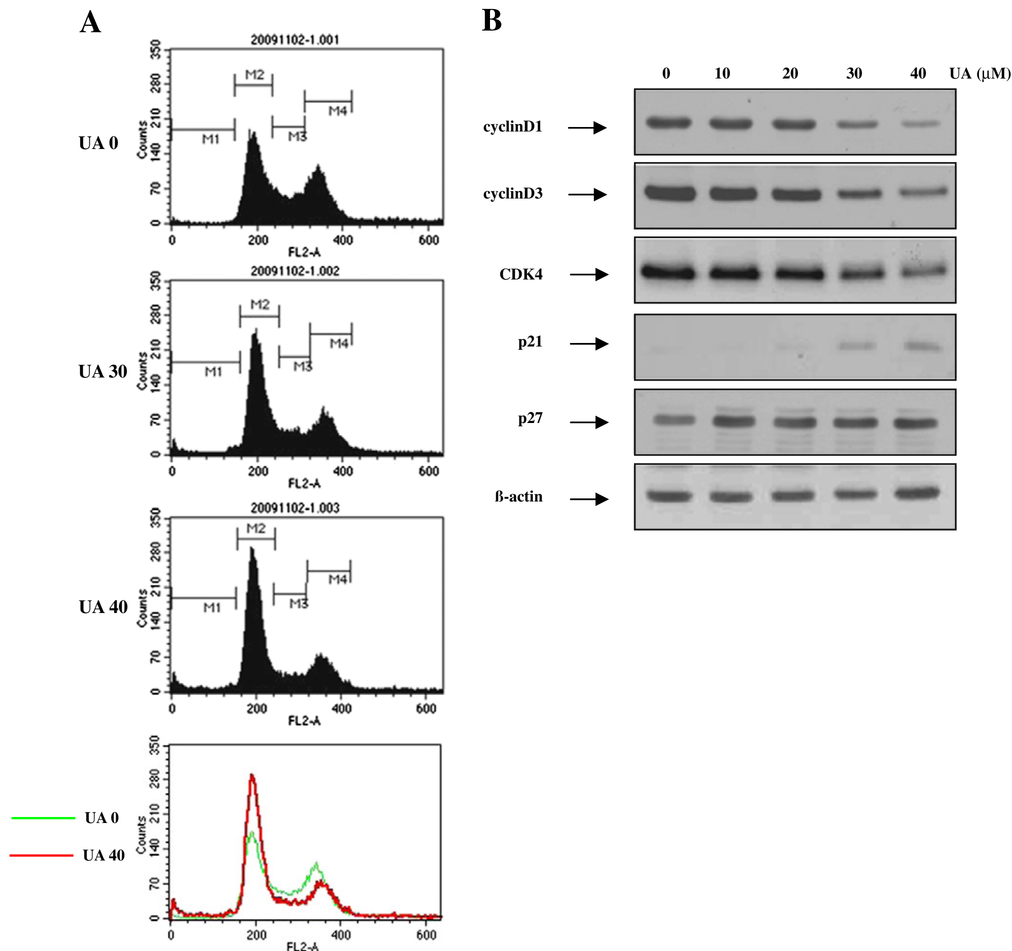

Fig. 2. UA resulted in G_1 arrest in PC3 cells. (A) DNA content analysis of PC3 cells treated with either vehicle (green) or 40 μM UA (red). (B) Immunoblot analysis of cell cycle markers. PC3 cells were treated with various concentrations of UA for 24 h. Total cell extracts were probed with antibodies against cyclin D1, D3, CDK4, p21, p27, and β-actin (loading control) as indicated.

3.3. UA triggers autophagy in PC3 cells

We verified that UA triggers G_1 phase arrest however, it did not induce apoptosis in the early stages of UA-induced cell death. Recent investigations indicate that chemo-therapeutic drugs, or any of several other anticancer stimuli such as aromatic amine p-anilinoaniline, induce autophagy but not apoptosis in various cancer cells [35]. Furthermore, the cytotoxicity of many anticancer agents is mediated by autophagy activation, which is associated with G_1 arrest [34,36,37]. The process of autophagy starts with the autophagosome formation and then progresses to autophagolysosomes through the fusion of acidic lysosomes with autophagosomes [38]. In order to verify whether UA induced the autophagic pathway, acridine orange staining of the live cells was employed to visualize acidic autophagolysosomes in control and UA-treated PC3 cells. As shown in Fig. 3A, UA treatment markedly elevated the amount of autophagolysosomes in the cells. Furthermore, we measured the incorporation of MDC, a marker for the acidic compartment of autolysosomes [37]. It is known that MDC accumulates in mature autophagic vacuoles (AVs) such as autophagolysosomes, but not in the early endosome compartment [21]. When cells were observed with a fluorescence microscope, AVs stained by MDC appear as distinct dot-like structures distributed within the cytoplasm or localizing in the perinuclear regions. As shown in Fig. 3B, there was an increase in the number of MDC-labeled vesicles at 24 h after the UA treatment. The results indicated an induction of AV formation by UA. To obtain better insight into the mechanism of UA-induced autophagy, we next analyzed the effects of UA on LC3-II protein, the lipidated form of mammalian microtubule-associated protein 1 light chain LC3-I. LC3-II is produced during autophagosome formation and the accumulation of LC3-II is considered one of the hallmarks of autophagy [22,39]. Using Western blot analysis with the anti-LC3 antibody, we examined the expressions of LC3-I (18 kDa) and LC3-II (16 kDa) in PC3 cells after treatment with various concentrations of UA. As shown in Fig. 3C, UA treatment of PC3 cells resulted in strong up-regulation of the LC3-II protein. Induction of autophagy by UA was also demonstrated by monitoring PC3 cells transfected with GFP–LC3 expressing vector. Fluorescence microscopy revealed a diffuse localization of GFP–LC3 in control cells. In contrast, treatment of cells with UA for 24 h produced punctas of GFP–LC3 fluorescence (Fig. 3D).

3.4. UA on mTOR signaling in PC3 cells

Our data indicate that UA induces both G_1 cell cycle arrest and autophagy. The common link of these two processes is that they can be modulated by mTOR signaling. mTOR, a PI3K-related serine/threonine kinase, plays a central role in regulating cell growth, proliferation, and survival, partly by regulation of translation initiation through interactions with other proteins such as raptor (forming mTOR complex 1, mTORC1) and rictor (forming mTOR complex 2, mTORC2). mTORC1 is primarily activated by the PI3K/Akt pathway [40,41]. PI3K–Akt activation is well-known to suppress autophagy in mammalian cells [18,31,42,43]. Activated mTOR is generally believed to be involved in the negative control of mammalian autophagy, although the mTOR effectors that regulate autophagy have not been clearly elucidated [39,44,45]. Rapamycin, an mTOR inhibitor, exerted its antitumor effect by inducing autophagy, suggesting that it disrupts the PI3K/Akt signaling pathway in malignant cancer cells [46]. Ceramide was shown to trigger autophagy by interfering with the mTOR signaling pathway [47,48]. We examined the role of the Akt/mTOR signaling pathway in UA-induced autophagy. UA treatment of PC3 cells resulted in a marked reduction of phosphorylation of Akt. UA did not cause any change in the protein levels of total Akt (Fig. 4A). In addition, treatment of UA decreased the phosphorylation

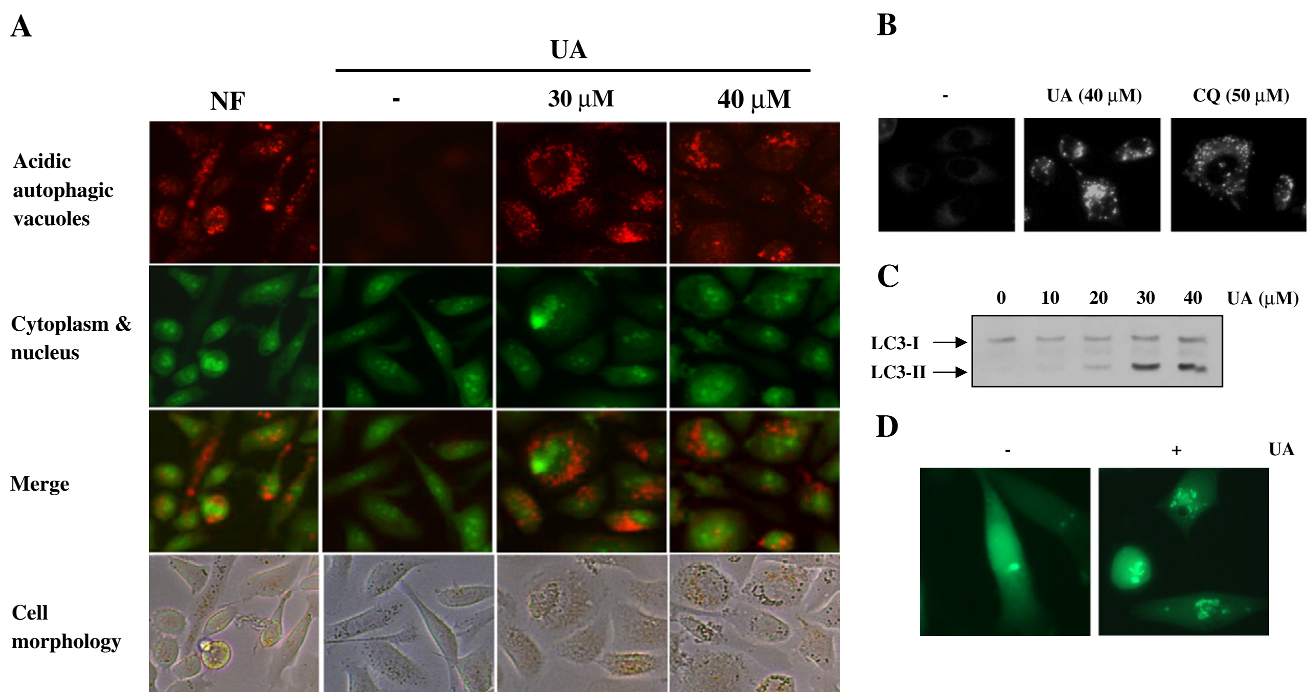

Fig. 3. UA induced autophagy in PC3 cells. (A) UA-induced formation of autophagic vacuoles in PC3 cells. Acridine orange staining was used to detect the autophagic vacuoles and formation of autophagic vacuoles in PC3 cells treated in the nutrient-free condition or treated with 40 μM UA for 24 h. Under acridine orange staining, the cytoplasm and nucleolus fluoresce green, whereas the acidic compartments fluoresce bright red or orange-red. (B) PC3 cells were treated with 40 μM UA for 24 h and staining MDC. Under MDC staining, mature autophagic vacuoles, such as autophagolysosomes, were observed. MDC staining of cells treated with chloroquine (CQ), an antimalarial agent that increases autophagosomal number, was presented as a positive control. (C) Total cell extracts were assayed by immunoblot analysis for expression of LC3-I and LC3-II. (D) Analysis of LC3 aggregation in UA treated cells. PC3 cells transiently transfected with GFP–LC3 using Lipofectamine 2000 (Invitrogen) were cultured in RPMI with 10% FBS in absence or presence of 40 μM UA for 24 h. Accumulation of GFP–LC3 puncta was observed in UA-treated transient transfectant.

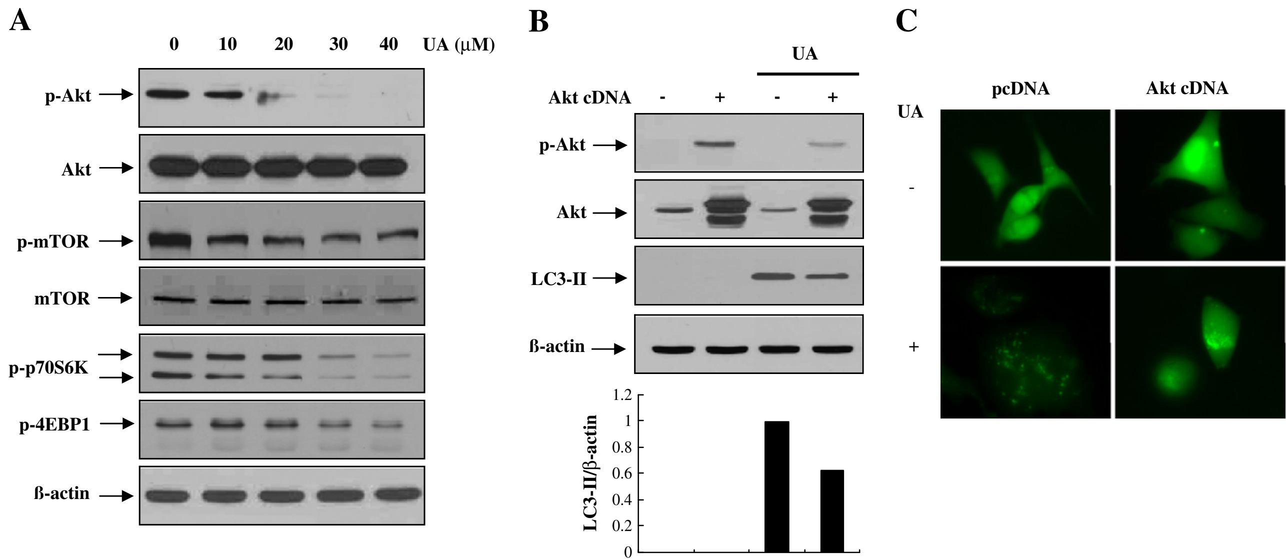

Fig. 4. UA treatment impacted on the mTOR signaling pathway. (A) Immunoblot analysis of the impact of vehicle or UA treatment of PC3 cells for 24 h on the levels of indicated proteins and phosphoproteins. (B) Influence of Akt overexpression by active Akt cDNA transfection on UA-mediated autophagy. Total cell extracts were assayed by immunoblot analysis for expression of LC3-II in Akt cDNA-transfected cells treated with or without UA. LC3-II bands were normalized to β-actin. (C) Effect of active Akt cDNA transfection on UA-induced LC3 translocation. PC3 cells transiently transfected with GFP–LC3 were pretreated or not with 5 mM 3-MA for 1 h and further treated with 40 μM UA for 24 h to observe the translocation of GFP–LC3.

of mTOR and its downstream targets including the 70 kDa ribosomal S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Fig. 4A). Both proteins play an important role in translational regulation; in particular, inhibition of expression of G_1 cell cycle regulatory protein cyclin D1 leads to G_1 arrest in cells in which the mTOR was inhibited [49]. To confirm the

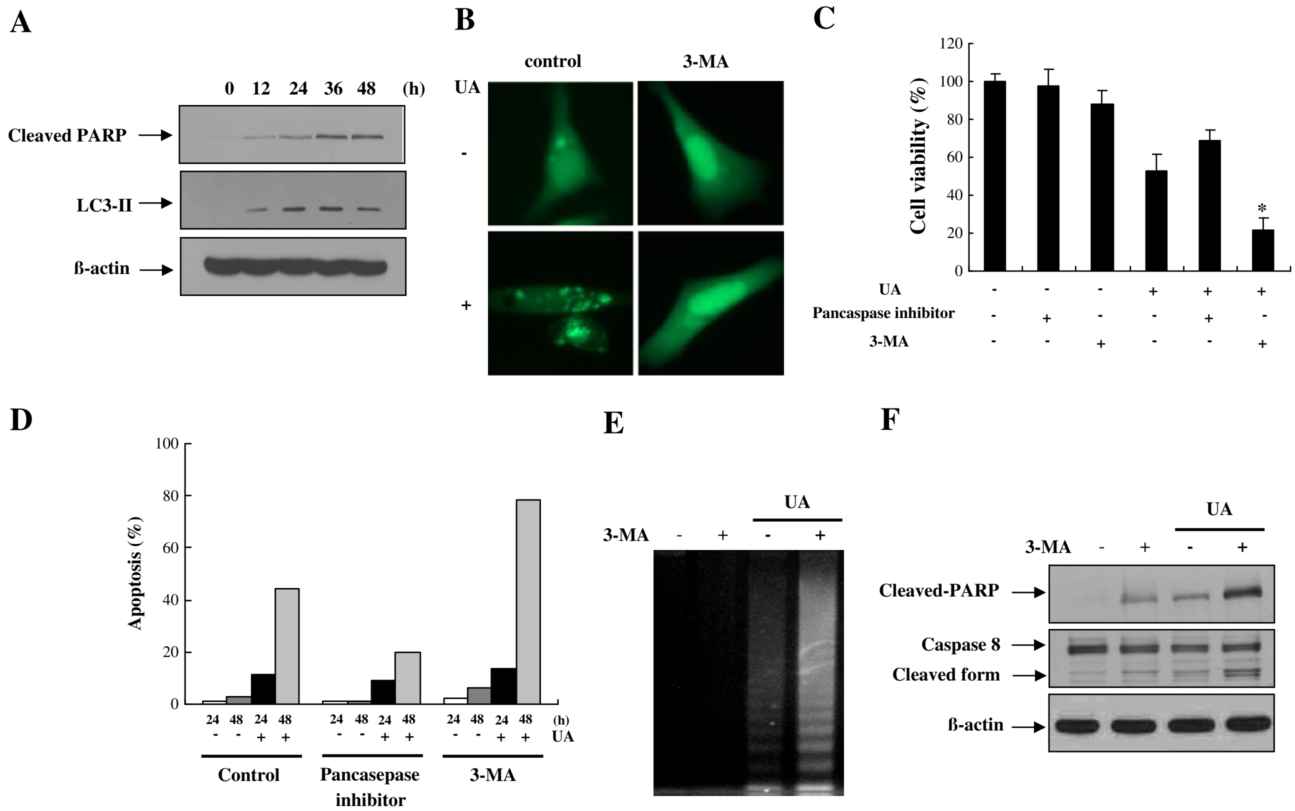

Fig. 5. Autophagy inhibition by 3-MA enhanced UA-induced apoptosis of PC3 cells. (A) Total cell extracts were assayed for immunoblot analysis for expression of cleaved PARP and LC3-II in PC3 cells treated with 40 μM UA for 0–48 h. (B) Effect of 3-MA on UA-induced LC3 translocation. PC3 cells transiently transfected with GFP–LC3 were pretreated or not with 5 mM 3-MA for 1 h and further treated with 40 μM ursolic acid for 24 h to observe the translocation of GFP–LC3. Representative images of the cells observed under fluorescence microscopy. (C) The viability of PC3 cells was measured by MTT analysis following 48 h of culture with UA with and without the autophagy inhibitor 3-MA. Data are presented as means ± S.D. of three separate experiments. *$p<0.01$ versus PC3 cells exposed to UA alone. (D) Apoptotic cell death was calculated from representative flow histograms depicting subdiploid fraction in PC3 cells treated for 24 h or 48 h with control or 40 μM UA in the presence or absence of 5 mM 3-MA. (E) Nuclear DNA fragmentation in PC3 cells treated for 48 h with control or 40 μM UA in the presence or absence of 5 mM 3-MA. (F) After the cells were exposed to 40 μM UA with and without 3-MA for 48 h, immunoblot analysis for cleaved PARP and caspase 8 was performed.

central role of the Akt signaling pathway as a target of UA-induced autophagy, we transfected PC3 cells with constitutively active form of Akt cDNA (Fig. 4B). Transfected cells expressing active Akt cDNA were considerably more resistant to UA-induced autophagy than cells transfected with the control cDNA. UA was notably unable to cause autophagy, which is reflected by attenuated accumulation of LC3-II, in cells transfected with active Akt cDNA, whereas UA could maintain its autophagy effects on PC3 transfected with the control cDNA (Fig. 4B). Similar results were also observed with UA-induced LC3 translocation in cells transfected with active Akt cDNA (Fig. 4C). Therefore, it can be firmly concluded that UA induces autophagy in PC3 cells by suppression of the Akt/mTOR pathway. Based on this data, we propose that UA leads to a consequent inhibition of Akt and mTOR signaling, which contributes to the effects on cell cycle progression and autophagy.

3.5. Autophagy counteracts UA-induced apoptosis

As mentioned above, we found that UA induced autophagy in early stage of the UA-induced cell death. In agreement with the above data, although UA-treated cells were arrested at the G_1 phase, but these cells did not undergo apoptosis at 24 h. Time-course of cleaved PARP and LC3-II expression also showed that autophagy proceeded apoptosis (Fig. 5A). To elucidate the involvement of the autophagic process in UA-induced apoptosis, the effects of autophagy inhibitor were tested. The UA-induced LC3 translocation was inhibited by 3-MA, a specific inhibitor of autophagy [50] (Fig. 5B), and cell viability was markedly decreased (Fig. 5C). In addition, the effect of 3-MA on UA-induced apoptosis was determined by analysis of sub-diploid cells by flow cytometry following staining with PI. Results showed that 3-MA alone had little effect on sub-G_1 fraction, an indicator of apoptotic cell death; however, pretreatment of PC3 cells with 3-MA significantly increased the UA-induced sub-G_1 apoptotic population (Fig. 5D). These results suggest that autophagy is a protective mechanism in the context of PC3 cells that perhaps allowed cells to escape from apoptosis. The 3-MA-mediated augmentation of UA-induced apoptosis was confirmed by analysis of DNA fragmentation, cleavage of PARP, and activation of caspase 8. As can be seen in Fig. 5E and F, both DNA fragmentation, cleavage of PARP, and activation of caspase 8 were significantly higher in PC3 cells co-treated with 3-MA and UA compared with that observed on treatment with UA alone. We also employed a knockdown strategy to impair autophagy in order to further assess its impact on UA-induced cell death. To investigate the specific functional role of key autophagy proteins Beclin-1 and Atg5 in UA-induced autophagy, we used an RNA interference approach for their specific inhibition. The silencing of Beclin 1 and Atg5 using their respective siRNA markedly induced cell death elicited by UA treatment (Fig. 6A and B). Knockdown of either Beclin-1 or Atg5 expression also resulted in the reduction of LC3-II production and accumulation of cleaved PARP in UA-treated cells (Fig. 6C and D), confirming a direct impact of knockdown on the autophagic process in the cells and consequent augmentation of apoptosis. Collectively, these studies suggest that the induction of autophagy provided a prosurvival role and delayed cell death during UA-induced injury to PC3 cells. Depending on the cellular context and/or initiating stimulus, autophagy may act as a protective mechanism for malignant cells or exhibit opposing effects and promote generation of antineoplastic responses [51]. Autophagy has been regarded as a tumor repression mechanism [17,34]. In contrast, recent studies report that inhibition of the autophagy exacerbated apoptosis, indicating that autophagy may be a protective response against anti-cancer agents that contributes to tumor progression [17,52-54]. In this aspect, the autophagic pathway is a novel therapeutic target for cancer treatment [52,54]. Autophagy inhibition enhances the anticancer effect of arsenic trioxide [19], hyperthermia [19], sulforaphane [55], and p53 or alkylating drugs [56]. These studies demonstrate that autophagy promotes cellular survival during UA-induced preapoptotic lag phase, resulting in delayed apoptotic response. Induction of autophagy during the initial period of UA insult may provide an appropriate environment to maintain cellular homeostasis before

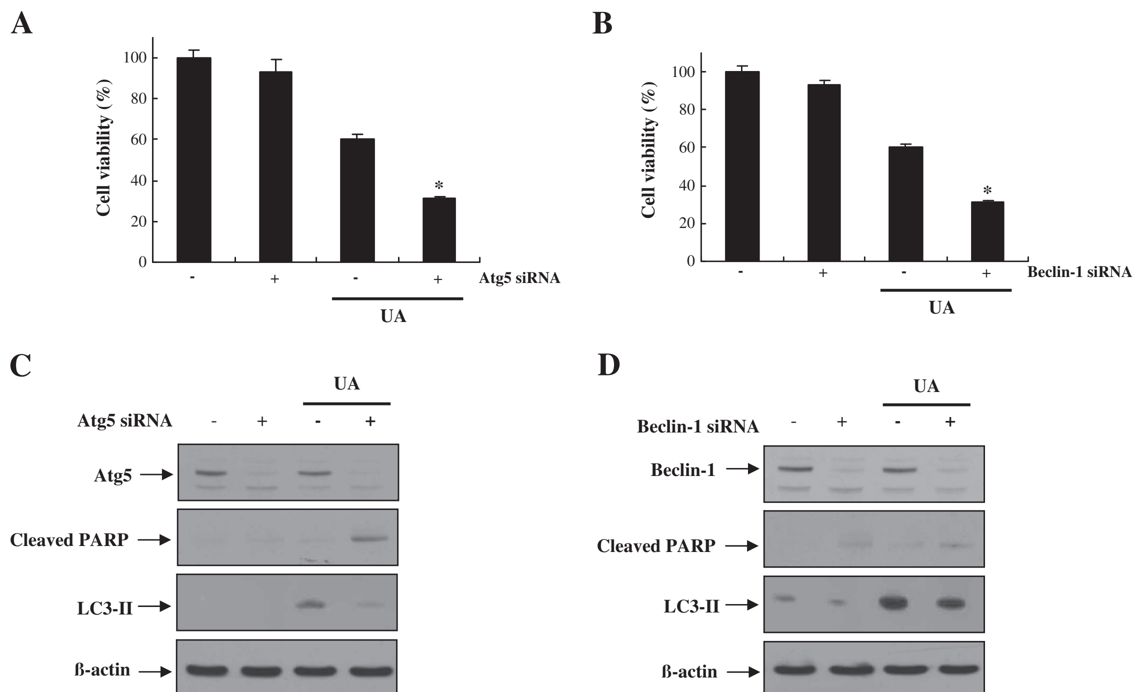

Fig. 6. Autophagy inhibition by siRNAs against Beclin-1 and Atg5 enhanced UA-induced apoptosis of PC3 cells. (A–B) PC3 cells were transfected with control siRNA or siRNAs specifically targeting Beclin-1 or Atg5 as indicated. Cells were then treated for 48 h with 40 μM UA, and the viability of PC3 cells was measured by MTT analysis. Data are presented as means ± S.D. of three separate experiments. $^{*}p<0.01$ versus the scrambled siRNA-transfected PC3 cells exposed to UA. (C–D) PC3 cells were transfected with control siRNA or siRNA specifically targeting Beclin-1 or Atg5 as indicated. Cells were then treated for 48 h with 40 μM UA, and total lysates were resolved by SDS-PAGE and immunoblotted with antibodies against Beclin-1, Atg5, cleaved PARP, LC3, and β-actin as indicated.

reaching the threshold for UA-induced apoptosis. Thus, at the initial stages of UA injury, autophagy may function efficiently in eliminating unwanted or damaged organelles and other cytoplasmic macromolecules to establish cellular homeostasis.

4. Conclusions

This study shows that UA produces in vitro growth inhibition, G_1 cell cycle arrest, and autophagy in the early stage of UA-induced cell death. Autophagy offers protection from the anti-cancer activity of UA in PC3 cells, and a combination of UA with autophagy inhibitors strengthens the therapeutic efficacy based on proapoptotic strategies. Collectively, this study indicates that a combination of UA with autophagy inhibitors might be essential in overcoming prostate cancer cell resistance. Furthermore, the approach may be an effective therapeutic strategy, in general, to sensitize therapy resistant cancer cells.

Acknowledgement

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2011-0001256).

References

[1] M.L. Gonzalo, W.B. Isaacs, Molecular pathways to prostate cancer, J. Urol. 170 (2003) 2444–2452.
[2] M.J. Hayat, N. Howlader, M.E. Reichman, B.K. Edwards, Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) program, Oncologist 12 (2007) 20–37.
[3] R.J. Long, K.P. Roberts, M.J. Wilson, C.J. Ercole, J.L. Pryor, Prostate cancer: a clinical and basic science review, J. Androl. 18 (1997) 15–20.
[4] D.P. Petrylak, Chemotherapy for advanced hormone refractory prostate cancer, Urology 54 (1997) 30–35.
[5] L.L. Pisters, The challenge of locally advanced prostate cancer, Semin. Oncol. 26 (1999) 202–216.
[6] J.P. Richie, Anti-androgens and other hormonal therapies for prostate cancer, Urology 54 (1999) 15–18.
[7] J. Liu, Pharmacology of oleanolic acid and ursolic acid, J. Ethnopharmacol. 49 (1995) 57–68.
[8] P.O. Harmand, R. Duval, B. Liagre, C. Jayat-Vignoles, J.L. Beneytout, C. Delage, A. Simon, Ursolic acid induces apoptosis through caspase-3 activation and cell cycle arrest in HaCat cells, Int. J. Oncol. 23 (2003) 105–112.
[9] B.B. Aggarwal, S. Shishodia, Molecular targets of dietary agents for prevention and therapy of cancer, Biochem. Pharmacol. 71 (2006) 1397–1421.
[10] C. Tang, Y.H. Lu, J.H. Xie, F. Wang, J.N. Zou, J.S. Yang, Y.Y. Xing, T. Xi, Downregulation of survivin and activation of caspase-3 through the PI3K/Akt pathway in ursolic acid-induced HepG2 cell apoptosis, Anticancer Drugs 20 (2009) 249–258.
[11] S. Prasad, V.R. Yadav, R. Kannappan, B.B. Aggarwal, Ursolic acid, a pentacyclin triterpene, potentiates TRAIL-induced apoptosis through p53-independent up-regulation of death receptors: evidence for the role of reactive oxygen species and JNK, J. Biol. Chem. 286 (2010) 5546–5557.
[12] M.T. Huang, C.T. Ho, Z.Y. Wang, T. Ferraro, Y.R. Lou, K. Stauber, W. Ma, C. Georgiadis, J.D. Laskin, A.H. Conney, Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid, Cancer Res. 54 (1994) 701–708.
[13] H. Cha, M.T. Park, H.Y. Chung, N.D. Kim, H. Sato, M. Seiki, K.W. Kim, Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells, Oncogene 16 (1998) 771–778.
[14] H. Yamai, N. Sawada, T. Yoshida, J. Seike, H. Takizawa, K. Kenzaki, T. Miyoshi, K. Kondo, Y. Bando, Y. Ohnishi, A. Tangoru, Triterpenes augment the inhibitory effects of anticancer drugs on growth of human esophageal carcinoma cells in vitro and suppress experimental metastasis in vivo, Int. J. Cancer 125 (2009) 952–960.
[15] E.H. Baehrecke, How death shapes life during development, Nat. Rev. Mol. Cell Biol. 3 (2002) 779–787.
[16] F. Reggiori, D.J. Klionsky, Autophagosomes: biogenesis from scratch? Curr. Opin. Cell Biol. 17 (2005) 415–422.
[17] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell 6 (2004) 463–477.
[18] K. Degenhardt, R. Mathew, B. Beaudoin, K. Bray, D. Anderson, G. Chen, C. Mukherjee, Y. Shi, C. Gelinas, Y. Fan, D.A. Nelson, S. Jin, E. White, Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis, Cancer Cell 10 (2006) 51–64.
[19] Y. Kondo, T. Kanzawa, R. Sawaya, S. Kondo, The role of autophagy in cancer development and response to therapy, Nat. Rev. Cancer 5 (2005) 726–734.
[20] S. Paglin, T. Hollister, T. Delohery, N. Hackett, M. McMahill, E. Sphicas, D. Domingo, J. Yahalom, A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles, Cancer Res. 61 (2001) 439–444.
[21] D.B. Munafo, M.I. Colombo, A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation, J. Cell Sci. 114 (2001) 3619–3630.
[22] Y.N. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[23] K. Fujiwara, S. Daido, A. Yamamoto, R. Kobayashi, T. Yokoyama, H. Aoki, E. Iwado, N. Shinojima, Y. Kondo, S. Kondo, Pivotal role of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in apoptosis and autophagy, J. Biol. Chem. 283 (2008) 388–397.
[24] M.B. Azad, Y. Chen, E.S. Henson, J. Cizeau, E. McMillan-Ward, S.J. Israels, S.B. Gibson, Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3, Autophagy 4 (2008) 195–204.
[25] L.C. Cantley, The phosphoinositide 3-kinase pathway, Science 296 (2002) 1655–1657.
[26] K.M. Nicholson, N.G. Anderson, The protein kinase B/Akt signalling pathway in human malignancy, Cell. Signal. 14 (2002) 381–395.
[27] Y. Lu, H. Wang, G.B. Mills, Targeting PI3K–AKT pathway for cancer therapy, Rev. Clin. Exp. Hematol. 7 (2003) 205–228.
[28] D. Morgensztern, H.L. McLeod, PI3K/Akt/mTOR pathway as a target for cancer therapy, Anticancer Drugs 16 (2005) 797–803.
[29] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-kinase–Akt pathway in human cancer, Nat. Rev. Cancer 2 (2002) 489–501.
[30] B.T. Hennessy, D.L. Smith, P.T. Ram, Y. Lu, G.B. Mills, Exploiting the PI3K/AKT pathway for cancer drug discovery, Nat. Rev. Drug Discov. 4 (2005) 988–1004.
[31] S. Arico, A. Petiot, C. Bauvy, P.F. Dubbelhuis, A.J. Meijer, P. Codogno, E. Ogier-Denis, The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway, J. Biol. Chem. 276 (2001) 35243–35246.
[32] N. Hay, The Akt–mTOR tango and its relevance to cancer, Cancer Cell 8 (2005) 179–183.
[33] M. Tanaka, D. Koul, M.A. Davies, M. Liebert, P.A. Steck, H.B. Grossman, MMAC1/PTEN inhibits cell growth and induces chemosensitivity to doxorubicin in human bladder cancer cells, Oncogene 19 (2000) 5406–5412.
[34] L. Longo, F. Platini, A. Scardino, O. Alabiso, G. Vasapollo, L. Tessitore, Autophagy inhibition enhances anthocyanin-induced apoptosis in hepatocellular carcinoma, Mol. Cancer Ther. 7 (2008) 2476–2485.
[35] A. Elliott, J.J. Reiners, Suppression of autophagy enhances the cytotoxicity of the DNA-damaging aromatic amine p-anilinoaniline, Toxicol. Appl. Pharmacol. 232 (2008) 169–179.
[36] J. Botti, M. Djavaheri-Mergny, Y. Pilatte, P. Codogno, Autophagy signaling and the cogwheels of cancer, Autophagy 2 (2006) 67–73.
[37] J. Liang, S.H. Shao, Z.X. Xu, B. Hennessy, Z. Ding, M. Larrea, S. Kondo, D.J. Dumont, J.U. Gutterman, C.L. Walker, J.M. Slingerland, G.B. Mills, The energy sensing LKB1–AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis, Nat. Cell Biol. 9 (2007) 218–224.
[38] M.M. Hippert, P.S. O'Toole, A. Thorburn, Autophagy in cancer: good, bad, or both? Cancer Res. 66 (2006) 9349–9351.
[39] D.C. Rubinsztein, J.E. Gestwicki, L.O. Murphy, D.J. Klionsky, Potential therapeutic applications of autophagy, Nat. Rev. Drug Discov. 6 (2007) 304–312.
[40] D.C. Fingar, J. Blenis, Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression, Oncogene 23 (2004) 3151–3171.
[41] P. Gulati, G. Thomas, Nutrient sensing in the mTOR/S6K1 signalling pathway, Biochem. Soc. Trans. 35 (2007) 236–238.
[42] A. Petiot, E. Ogier-Denis, E.F. Blommaart, A.J. Meijer, P. Codogno, Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells, J. Biol. Chem. 275 (2000) 992–998.
[43] D.J. Klionsky, A.J. Meijer, P. Codogno, Autophagy and p70S6 kinase, Autophagy 1 (2005) 59–60.
[44] T. Yorimitsu, D.J. Klionsky, Autophagy: molecular machinery for self-eating, Cell Death Differ. 12 (2005) 1542–1552.
[45] S. Pattingre, L. Espert, M. Biard-Piechaczyk, P. Codogno, Regulation of macroautophagy by mTOR and Beclin 1 complexes, Biochimie 90 (2008) 313–323.
[46] M. Castedo, K.F. Ferri, G. Kroemer, Mammalian target of rapamycin (mTOR): pro-and anti-apoptotic, Cell Death Differ. 9 (2002) 99–100.
[47] F. Scarlatti, C. Bauvy, A. Ventruti, G. Sala, F. Cluzeaud, A. Vandewalle, R. Ghidoni, P. Codogno, Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1, J. Biol. Chem. 279 (2004) 18384–18391.
[48] S. Pattingre, C. Bauvy, S. Carpentier, T. Levade, B. Levine, P. Codogno, Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy, J. Biol. Chem. 284 (2009) 2719–2728.
[49] M.S. Neshat, I.K. Mellinghoff, C. Tran, B. Stiles, G. Thomas, R. Petersen, P. Frost, J.J. Gibbons, H. Wu, C.L. Sawyers, Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 10314–10319.
[50] T. Shintani, D.J. Klionsky, Autophagy in health and disease: a double-edged sword, Science 306 (2004) 990–995.
[51] D.J. Goussetis, J.K. Altman, H. Glaser, J.L. McNeer, M.S. Tallman, L.C. Platanias, Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide, J. Biol. Chem. 285 (2010) 29989–29997.
[52] B. Levine, Autophagy and cancer, Nature 446 (2007) 745–747.
[53] R. Mathew, V. Karantza-Wadsworth, E. White, Role of autophagy in cancer, Nat. Rev. Cancer 7 (2007) 961–967.
[54] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (2008) 27–42.
[55] A. Herman-Antosiewicz, D.E. Johnson, S.V. Singh, Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells, Cancer Res. 66 (2006) 5828–5835.
[56] R.K. Amaravadi, C.B. Thompson, The roles of therapy-induced autophagy and necrosis in cancer treatment, Clin. Cancer Res. 13 (2007) 7271–7279.