Pharmacological ascorbate induces cytotoxicity in prostate cancer cells through ATP depletion and induction of autophagy

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Recent studies have revealed the scientific basis for the use of intravenous (i.v.) vitamin C or ascorbic acid (ascorbate) in treating cancers, and raised the possibility of using i.v. ascorbate as a prooxidant anticancer therapy. Through the production of H_2O_2, pharmacologic ascorbate can induce some cancer cell death *in vitro* and inhibit a number of types of tumor growth in animal models. However, the mechanism of cell death triggered by ascorbate is not well understood. In this study, we investigated the cytotoxicity of pharmacological concentrations of ascorbate to human prostate cancer cells and the mechanisms involved. The results showed that ascorbate in the millimolar range induced cytotoxicity in five of the six tested prostate cancer cell lines. The IC_{50} values in the sensitive prostate cancer cells ranged from 1.9 to 3.5 mmol/l, concentrations clinically achievable with i.v. ascorbate use. All tested androgen-independent cells were sensitive to ascorbate treatment. The ascorbate-insensitive cell line LaPC4 is hormonally dependent. Whereas the reasons for sensitivity/resistance to ascorbate treatment need to be investigated further, cell death in sensitive cells was dependent on H_2O_2. Ascorbate treatment depleted ATP and induced autophagy in sensitive prostate cancer cells, resulting in cell death. Taken together with previous studies, high-dose ascorbate has the potential to be a novel treatment option to hormone-refractory prostate cancer. *Anti-Cancer Drugs* 23:437–444 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among men in the US [1]. One man in six will be diagnosed with prostate cancer during his lifetime, and one man in 35 will die of this disease [1]. Although prostate cancer patients diagnosed in the early stages can be treated successfully by standard treatments including prostatectomy, androgen-deprivation therapy, chemotherapy, radiation therapy, and cryoablation, there are still no effective treatments for hormone-refractory prostate cancer (HRPC), and for those who have advanced or metastatic disease or for those who have failed the primary curative attempts. The prognosis is dismal for these patients. Novel and improved treatments for this common disease are needed.

Intravenous (i.v.) vitamin C or ascorbic acid (ascorbate) has been used in cancer treatment for many years in complementary, alternative, and integrative medicine practices, with anecdotal efficacy and unclear mechanisms [2–9]. Until recently, the extent of i.v. ascorbate use was unknown [10]. From the reported survey data, it is now known that i.v. ascorbate is in wide use and doses up to 200 g per infusion are used to treat a variety of conditions including cancer. The i.v. ascorbate infusions are remarkably safe, with very few adverse events reported in ~20 000 patients over a period of 2 years [10]. Recently, accumulating evidence revealed the basis of this use and showed that high-dose i.v. ascorbate may play a therapeutic role in cancer. When taken orally, plasma concentrations of ascorbate are tightly controlled at less than 100 μmol/l by limited bioavailability and renal excretion [11]; i.v. ascorbate bypasses this tight control and induces a very different mechanism than oral ascorbate. Concentrations in the millimolar range can be produced by an i.v. ascorbate infusion, which cannot be achieved by maximum oral doses [12]. These pharmacologic ascorbate concentrations result in the production of H_2O_2 in the extracellular space [13–15], and then result in cytotoxicity in cancer cells, with a much lower sensitivity in normal cells [14–17]. These plasma concentrations of ascorbate are attainable in human clinical trials [14,18]. So far, a number of animal tumor models have been tested and pharmacologic ascorbate has been shown to reduce tumor growth. These include glioblastoma, pancreatic cancer, ovarian cancer, hepatoma, colon cancer, sarcoma, leukemia, mesothelioma, and neuroblastoma [13–17,19–24]. In 2010, Pollard *et al.* [16] reported that a high dose of intraper-

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itoneal ascorbate suppressed a HRPC in a syngeneic rat model. Taken as a whole, i.v. ascorbate is a prodrug for H_2O_2 production in the extracellular space, resulting in targeted neoplastic cell death.

In the current study, we further investigated the cytotoxicity of ascorbate to different types of prostate cancer cell lines, including androgen-dependent and androgen-independent cells. Moreover, we will investigate the mechanism(s) of ascorbate-induced cell death, which has not been well understood. Although some studies have shown apoptosis in ascorbate-induced cell death in some types of cancer cells [25–27], more recent studies showed that ascorbate-induced cell death was not mainly apoptosis and was caspase independent [19,28–31]. Some studies have suggested that a new form of cell death, autoschizis, was the major form of cell death induced by an ascorbate/menadoine combination, which was caspase independent and was characterized by membrane damage and enucleation, cell organ damage, and nucleolar segregation and chromatin decondensation [32–35]. The morphologies of autoschizis showed both apoptotic and necrotic characteristics [33,36]. Still, some studies showed that ascorbate alone was able to induce autophagy in a pancreatic cancer cell line [17,37]. The goal of this study is to determine whether ascorbate is cytotoxic to various kinds of prostate cancer cells, and if so, to determine the mechanism of ascorbate-induced cytotoxicity. We hypothesized that H_2O_2 formed by ascorbate will deplete cell ATP and will induce autophagy in prostate cancer cells that leads to cell death.

Materials and methods

Cell treatment and viability assay

Prostate cancer cell lines PC-3, LNCap, C4-2, LaPC4, and 22 RV1 were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and were cultured in recommended media. PC-3/GFP-LC3 was established in Dr Li's lab at the University of Kansas Medical Center by stably transfecting PC-3 cells with the GFP-labeled *LC3* gene [38]; PC-3/shBif1 cells were established by transfecting Bif-1 shRNA as previously described [38–40]. These cells were cultured in RPMI 1640 (Sigma, St Louis, Missouri, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Serial dilutions of ascorbate were incubated with cells for 2 h, and were then washed out to mimic clinically relevant conditions. Cells were continually incubated in fresh media until viability was detected after 24 h. To examine the contribution of H_2O_2 toward ascorbate-induced cell death, 600 U/ml of catalase was added before ascorbate incubation. Relative viability was detected by a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, on the basis of the ability of living, but not dead cells to reduce MTT to formazan [41,42]. Half-inhibitory concentration (IC_{50}) was defined as the concentration of drug that inhibited cell growth by 50% relative to untreated control. Ascorbate (Sigma) was prepared as 1 mol/l stock solutions in sterile water, with sodium hydroxide added drop-wise to adjust the pH to 7.0. Aliquots stored frozen at −80 °C were thawed for single use.

Cellular ATP detection by high-performance liquid chromatography

As high concentrations of ascorbate interfere with the commonly used luciferase-based ATP assays, high-performance liquid chromatography (HPLC) was utilized for the detection of ATP using a modified protocol on the basis of a reported method [43]. Briefly, PC-3 cells in a six-well plate (2×10^5 cells/well) were treated with 5 mmol/l ascorbate. At 0, 0.5, 1, 2, and 6 h, cells were harvested using a rubber scraper, and were washed and resuspended in PBS. ATP were extracted by quickly lysing the cells in a 0.05 mol/l KOH solution, and then the mixture was immediately neutralized to pH 6 with 0.1 mol/l KH_2PO_4. After centrifuging it, the supernatant was analyzed using a gradient HPLC method on a Waters e2695 HPLC with ultraviolet detection at 254 and 340 nm (Waters 2489 diode array UV detector; Waters, Milford, Massachusetts, USA). Reversed-phase chromatography was performed with an XBridgeTM C18 column 3.5 µm (Waters). The mobile phase (pH 6) contained acetonitrile (2% for solvent A and 30% for solvent B), 0.1 mol/l KH_2PO_4, and 0.008 mol/l tetrabutylammonium hydrogen sulfate. The fractions of solvent A to solvent B at 0, 4, 7, 12, 15, and 22 min were 100 : 0, 90 : 10, 80 : 20, 60 : 40, 0 : 100, and 100 : 0, respectively. With 8 min postrun, the total run time was 30 min per sample. Empower II software (Waters) was used for instrument control and data analysis. All values were normalized to the protein content of the whole-cell lyate using the bicinchoninic acid method (Pierce Biotechnology, Rockford, Illinois, USA) [44].

Autophagy detection

PC-3 cells were transfected with the pGFP-LC3 plasmid and a stable transfection clone named PC-3/GFP-LC3 was established [38]. Cells were cultured in tissue culture-treated chamber glass slides (BD Falcon, Franklin Lakes, New Jersey, USA) and treated with 5 mmol/l of ascorbate. Fluorescence imaging was performed to detect the punctuate pattern of green fluorescent protein (GFP)-LC3, which is indicative of autophagy. The processing of LC3-I to LC3-II was detected using western blot. Whole-cell proteins were isolated from untreated and ascorbate-treated PC-3 and PC-3/GFP-LC3 cells, and 60 µg of protein was loaded for SDS-polyacrylamide gel electrophoresis. The antibodies were anti-LC3-B (Cell Signaling, Danvers, Massachusetts, USA) and anti-GFP (Cell Signaling), and anti-β-actin was used as a control.

Transmission electron microscopy

Cells were harvested using trypsin-EDTA, fixed in 2% glutaraldehyde for 4 h, and centrifuged to form pellets.

Sample preparation was carried out according to the previously reported method [45]. Briefly, the pellets were rinsed in 0.1 mol/l cacodylate buffer [Electron Microscopy Sciences (EMS), Hatfield, Pennsylvania, USA] and postfixed in 1% osmium tetroxide (EMS). Cell pellets were dehydrated through a graded series of ethanol, passed through propylene oxide twice and finally placed in propylene oxide/Embed 812 resin (EMS) overnight for infiltration, and then polymerized in a 60°C oven overnight. Then, sections were cut on a Leica UCT ultra microtome (Leica Microsystems, Buffalo Grove, Illinois, USA) at 80 nm using a Diatome diamond knife. Sections were contrasted with uranyl acetate and Sato's lead citrate (EMS), and viewed and photographed on a JEOL 100CXII TEM at 60 kV (JEOL Ltd, Tokyo, Japan).

Results

Ascorbate induced H_2O_2-dependent cytotoxicity in prostate cancer cells

Six different prostate cancer cell lines were tested for the cytotoxic effect of pharmacologic ascorbate. The origin and hormonal dependence of these cells are listed in Table 1. Cells were treated with 0–20 mmol/l of ascorbate for 2 h, which are relevant to clinical concentrations and time course with i.v. ascorbate usage [14,18]. Five out of the six tested cells were sensitive to ascorbate treatment, with IC_{50} values ranging from 1.9 (22 RV1 cells) to 3.45 mmol/l (C4-2 cells) (Fig. 1a), concentrations easily achievable from an i.v. ascorbate infusion [12]. All the hormonally tested independent cells are sensitive to ascorbate treatment. In the two androgen-dependent cells, one was sensitive (LNCap, $IC_{50} = 2.9$ mmol/l) and the other was resistant (LaPC4, $IC_{50} > 20$ mmol/l). In all the tested cell lines, the addition of catalase, a H_2O_2 scavenger to the culture media, completely protected the cells from the death caused by ascorbate (Fig. 1b). Consistent with previous studies, this indicated that the cell death was mediated by H_2O_2 formed from high concentrations of ascorbate [13,14,19].

Ascorbate treatment decreased ATP levels in prostate cancer cells

H_2O_2 can decrease ATP in sensitive cells by at least three mechanisms [13]. First, increased H_2O_2 causes DNA damage, repaired by poly-ADP-ribose polymerase (PARP). Enhanced poly-ADP-ribose polymerase activity may deplete NAD^+, resulting in a decrease in ATP synthesis [51,52]. Second, H_2O_2 detoxification in cells oxidizes glutathione, which is regenerated with reducing equivalents from NADPH, which in turn is regenerated from glucose through the pentose phosphate pathway. The glucose used to reduce $NADP^+$ to NADPH is not available for ATP generation [53–57]. Finally, cancer cell mitochondria may have increased sensitivity to H_2O_2 [56,58,59].

To determine whether ascorbate depletes ATP in sensitive cancer cells, PC-3 cells were treated with 5 mmol/l ascorbate, and ATP was detected at different time points from 0.5 to 6 h. A decrease in cellular ATP was detected as early as 0.5 h of treatment. At 2 h of treatment, ATP concentrations in the treated cells decreased to *22%* of those in the untreated cells (Fig. 2a). Direct comparison of the ATP contents showed that the decrease was significant from 0.5 till 6 h of the treatment (Fig. 2b). There was no restoration of cellular ATP levels within 6 h, at which time-point the earliest cell death was observed.

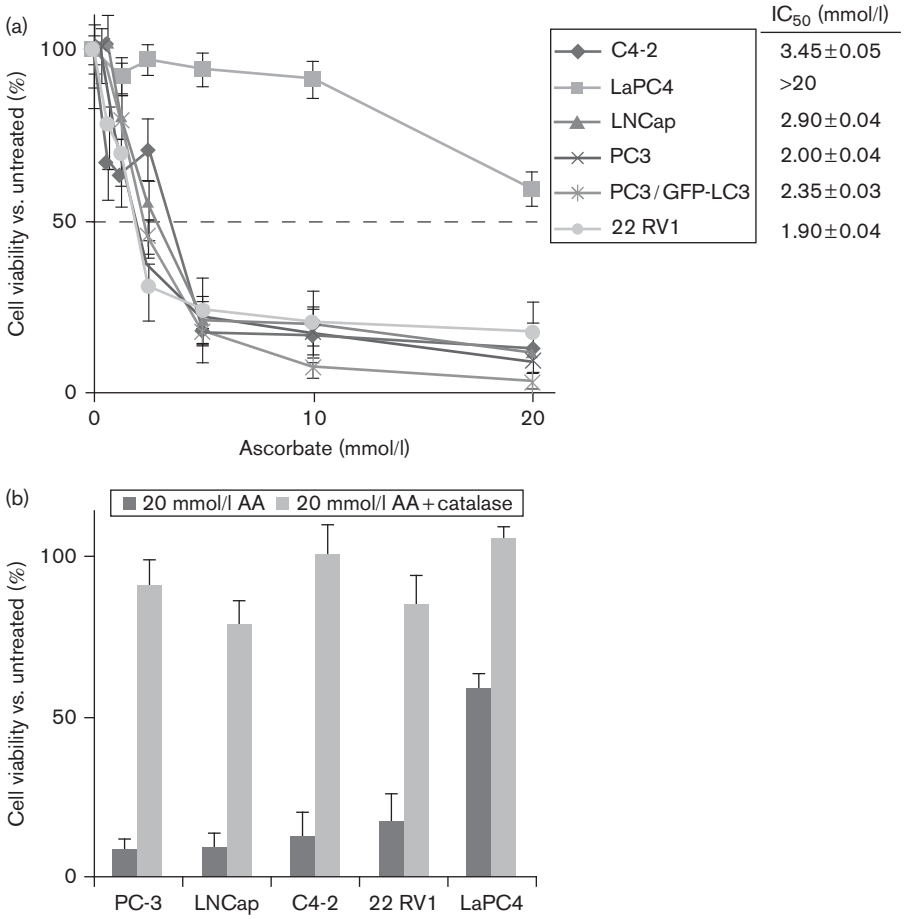
Ascorbate treatment induced autophagy in prostate cancer cells

Previous studies have indicated that ascorbate may lead to cell death through a unique caspase-independent autophagy pathway [29]. Activation of the autophagy pathway can be detected by the processing of LC3 to the lipidated form referred to LC3-II and the redistribution of LC3-II to the surface of autophagosomes. To test, we used western blot analysis to determine the amount of LC3-II in ascorbate-treated PC-3 cells. Increased levels of LC3-II were clearly detected in PC-3 cells exposed to 5 mmol/l of ascorbate (Fig. 3a). In addition, coincubation with lysosomal protease inhibitors chloroquine (CQ) and hydroxychloroquine (HCQ), which block the final steps of autophagic degradation, enhanced ascorbate-induced accumulation of LC3-II (Fig. 3a). In contrast, 3-methylamphetamine (3-MA), which inhibits autophagosome formation, markedly reduced the accumulation of LC3-II (Fig. 3a). We then treated the PC-3/GFP-LC3 cells with ascorbate. Fluorescence microscopy showed that GFP-LC3 signals shifted from a diffuse cytoplasmic pattern to a punctuate membrane pattern after 6 h of exposure to 5 mmol/l ascorbate, indicating that autophagic vacuoles were formed (Fig. 3b). Transmission electromicroscopy was performed and the formation of autophagosomes was clearly observed with ascorbate

Table 1 Origin and androgen dependence of the cell lines used

Cell line	Origin	Androgen dependence	Ref.
PC-3	Human prostate adenocarcinoma bone metastasis	Independent	[46]
PC-3/GFP-LC3	Derived from PC-3 by transfection of GFP-labeled *LC3* gene	Independent	[38]
22 RV1	Relapse of androgen-dependent CWR22 xenografts in castrated mice	Independent	[47]
C4-2	Subline derived from LNCap	Independent	[48]
LNCap	Prostate carcinoma lymph node metastasis	Dependent	[49]
LaPC4	Human prostate cancer xenograft in SCID mice	Dependent	[50]

Fig. 1



Ascorbate-induced H_2O_2-dependent cell death in prostate cancer cells. (a) Cells were exposed to serial dilutions of ascorbate (0–20 mmol/l) for 2 h, and then media were changed and cell viability was detected 24 h later. The IC_{50} values were determined for the concentrations that reduced viability by 50% compared with matched untreated cells. All data represent two to three individual experiments, each carried out in triplicate (±SD). (b). Before ascorbate exposure, catalase (600 U/ml) was added to the culture media, and then 20 mmol/l ascorbate was added and incubated with the cells for 2 h. Cells were washed and placed in fresh media. Cell viability was detected 24 h later. Data represent experiments carried out in six repeats (±SD). AA, ascorbate.

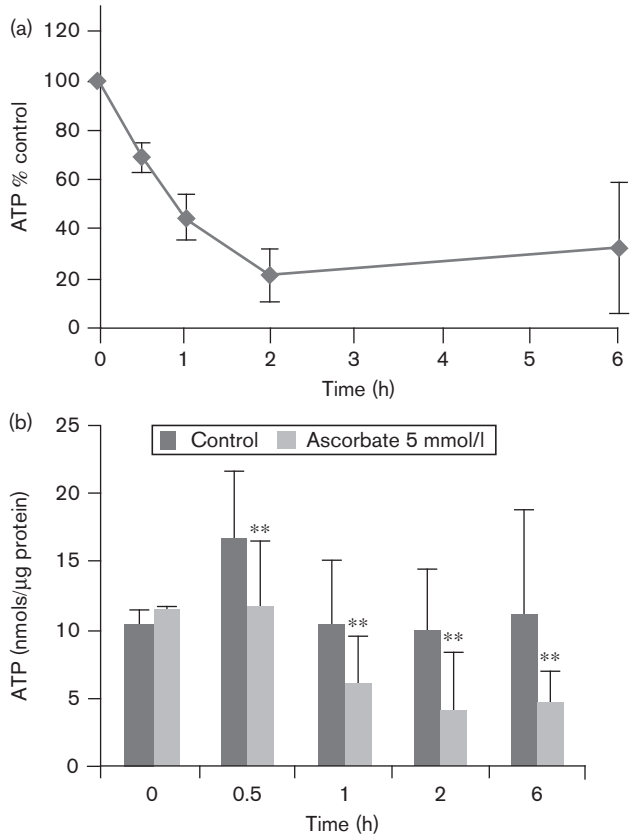
treatment (Fig. 3c). The percentage of cells containing autophagosomes increased from 5% in the untreated cells to 80% in ascorbate-treated cells. The number of autophagosomes per cell also increased markedly. Pretreatment with catalase largely attenuated the autophagy induced by ascorbate.

Remarkably, 3-MA not only inhibited LC3-II accumulation induced by ascorbate but also significantly inhibited the cell death caused by ascorbate (Fig. 3d). Moreover, as Bif-1 is a known positive mediator for autophagy [39], we obtained PC-3 cell lines with decreased Bif-1 expression (PC-3/shBif1) using shRNA targeting Bif-1, as previously described [38]. In the Bif-1 knocked-down cell line PC-3/shBif1, ascorbate-induced cell death was largely reduced compared with the parent PC-3 cells by either 2, 6, or 24 h of treatment (Fig. 3e). As autophagy may either induce cell death or act as a protective approach depending on the cell context, these data strongly suggested that ascorbate-induced autophagy resulted in cell death in sensitive prostate cancer cells.

Discussion

Ascorbate is one of the early unorthodox therapies for infectious disease and cancer since its introduction in the first half of the 20th century [2–4,7–9,60,61]. This approach was subsequently promoted by Cameron and Pauling [5,6,62,63]. Later, because of negative reports regarding its utility in chronic disease [64,65], ascorbate treatment has largely been abandoned by conventional medicine and has become a therapy primarily used by complementary, alternative, and integrative medicine practitioners [10,66]. However, recent *in vitro* and *in vivo* research findings support the use of high-dose i.v. ascorbate in cancer treatment and provide a mechan-

Fig. 2



Ascorbate reduced cellular ATP in PC-3 prostate cancer cells. PC-3 cells in the logarithm growth phase were treated with 5 mmol/l of ascorbate. At 0.5, 1, 2, and 6 h of treatment, cells were harvested with a rubber scrapper. Untreated cells were also harvested at the respective time point. Cellular ATP was detected with HPLC and normalized to the total protein content. (a) Percentage of ATP in treated cells compared with untreated cells at the matched time points. (b) Cellular ATP contents of treated and untreated cells at each matched time point. $^{**}P < 0.01$. Data represent two independent experiments, each carried out in triplicate (± SD).

ism for neoplastic cytotoxicity [14–17,19]. The i.v. ascorbate has a mechanism very different from that of oral ascorbate. Clinical data indicate that when ascorbate is given orally, plasma concentrations are tightly controlled at less than 100 μmol/l [11]. When doses exceed 250 mg, absorption decreases, urine excretion increases, and ascorbate bioavailability decreases [11,12]. In contrast, when 1.25 g of ascorbate is administered i.v., plasma concentrations of more than 1 mmol/l are achieved. With higher doses both i.v. and intraperitoneal administration of ascorbate can achieve plasma concentrations more than 20 mmol/l [14,18]. Recent survey data revealed that i.v. doses as high as 100 g are used in patients, and estimated plasma concentrations would reach around 30 mmol/l [10]. These high concentrations of ascorbate generate ascorbate radical in the extracellular space, which in turn reacts with a transition metal to produce H_2O_2 [13,14]. It is H_2O_2 that acts as the cytotoxic agent and as such, high-dose ascorbate is a prodrug for H_2O_2 with apparent targeted neoplastic cytotoxicity. Our data in this study confirm the prooxidant anticancer effect of ascorbate using clinically relevant conditions, as ascorbate-induced cell death was attenuated by the H_2O_2 scavenger catalase.

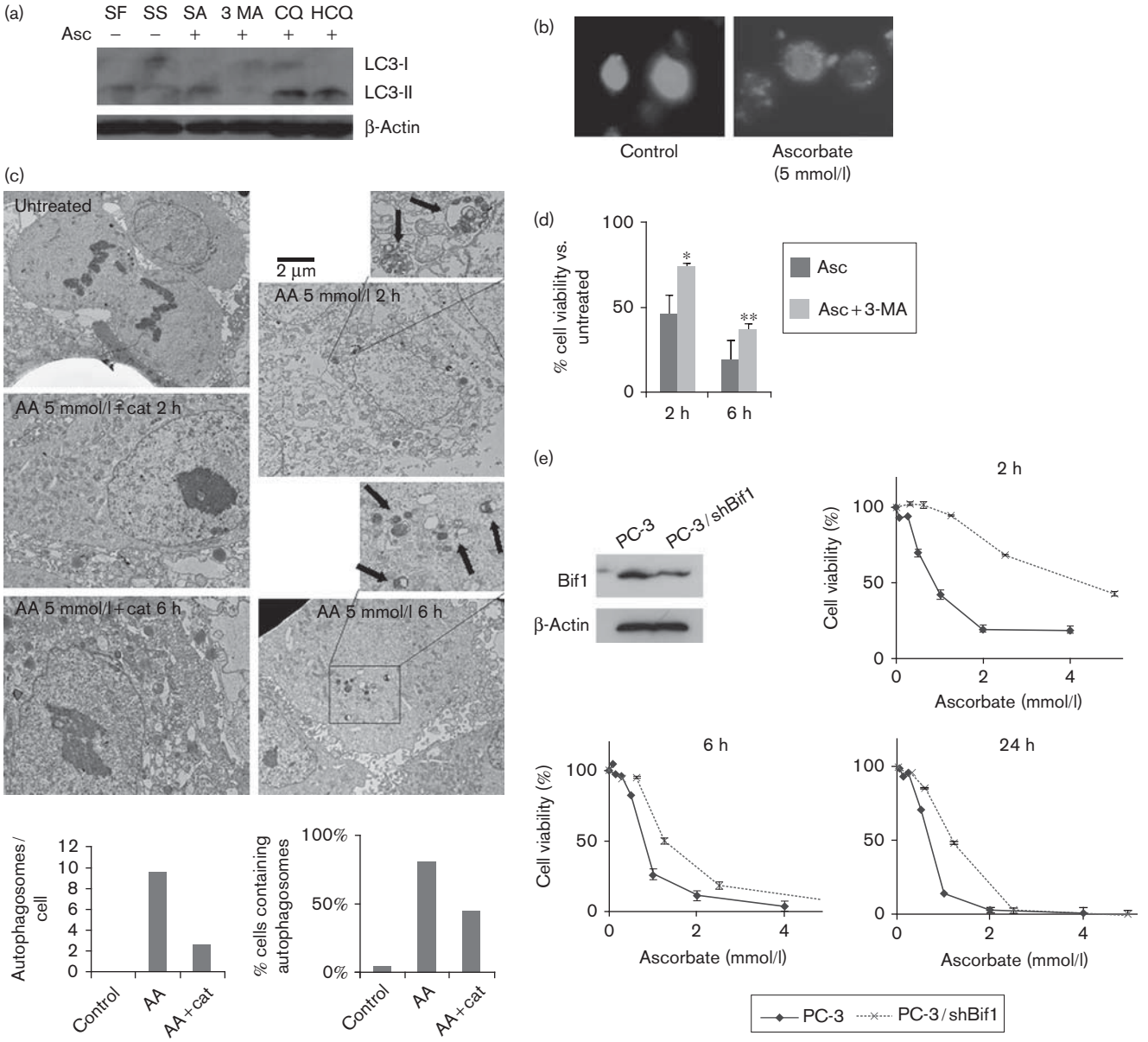
High-dose i.v. ascorbate is remarkably safe [10,18]. A recent survey report and a phase I clinical trial show that when patients have normal glucose-6-phosphate dehydrogenase activity and normal renal function, adverse events and toxicity are minimal even with i.v. doses as high as 1.5 g/kg, equivalent to 105 g for a 70 kg person [10,18]. Along with its highly suggestive anti-tumor efficacy and low toxicity and expense, there may be several advantages of using ascorbate as cancer treatment.

Prostate cancer is the most frequently occurring cancer among male patients, and has been estimated as the second most common cause of death among them in the USA during 2011 [1]. HRPC represents the most hard-to-treat condition in prostate cancer therapy. The median survival for men with metastatic HRPC is 1–2 years, with improvements in survival seen primarily with docetaxel-based therapies [67]. Treatment options are limited, and there is a clear need for therapies that improve outcome. In our study, ascorbate induced cell death in all the tested hormone-independent prostate cancer cells. The data indicate a potential for ascorbate to inhibit HRPC.

The depletion of ATP in sensitive cancer cells may be a potential mechanism for preferential cancer cell death unlike normal cells. Owing to mitochondrial dysfunction, hypoxia in the tumor microenvironment, and oncogenic signals, many malignant cells rely primarily on glycolysis for ATP production (the Warburg effect); thus, their ATP synthesis is not efficient compared with normal cells that primarily use oxidative phosphorylation [68–70]. Therefore, some cancer cells may be particularly sensitive to pharmacologic ascorbate concentration, compared with normal cells. Our data revealed a significant decrease in ATP shortly after cancer cells were exposed to ascorbate. Ascorbate-induced oxidative stress could represent a difference between tumor cell and normal cell metabolism that allows manipulations designed to improve therapy for cancer.

Our study also suggested that an autophagic mechanism may be involved in ascorbate-induced prostate cancer cell death, as LC3 processing and relocation was observed, and electromicroscopy clearly showed autophagic morphologies. The role of autophagy in cellular responses to oxidative stress can be a two-way effect. Reactive oxygen species (ROS) can induce autophagy, which contributes to caspase-independent cell death in a variety of cell types [71]. In contrast, a number of studies show a protective role of autophagy against ROS-mediated

Fig. 3



Ascorbate induced autophagy in PC-3 cells. (a) Western blots for LC-3II accumulation. PC-3 cells were treated with 5 mmol/l ascorbate for 2 h. SF, cells were starved in serum-free medium for 2 h without ascorbate treatment; SS, cells were in complete medium without any treatment; SA, cells were treated in complete medium with 5 mmol/l ascorbate; 3-methylamphetamine (3-MA), chloroquine (CQ), hydroxychloroquine (HCQ), 1 mmol/l 3-MA, 10 μmol/l CQ, or 10 μmol/l HCQ was coincubated with 5 mmol/l ascorbate, respectively. (b) PC-3/GFP-LC3 cells were treated with 5 mmol/l ascorbate for 6 h. GFP-LC3 punctuation was shown. (c) Electromicroscopy was performed after PC-3 cells were treated with 5 mmol/l ascorbate for 2 h and 6 h. Representative electomicroscopy pictures are shown. The cells containing autophagosomes were counted, and the number of autophagosomes was averaged to show autophagosomes per cell. At least 20 cells were counted in each group. Catalase (cat, 600 U/ml) was added before ascorbate treatment. Arrows point to typical autophagosomes. (d) 3-MA inhibited ascorbate-induced cell death. PC-3 cells were incubated with either 1.25 mmol/l ascorbate or ascorbate (1.25 mmol/l) plus 3-MA (1 mmol/l). At 2 h and 6 h of incubation, cells were washed and placed in fresh culture media. Cell viability was detected 24 h later. $*P<0.05$; $**P<0.01$. (e) Cytotoxicities of ascorbate in PC-3 cells and PC-3/shBif1 cells. Western blots showed that Bif-1 expression was inhibited in PC-3/shBif1 cells. PC-3 cells and PC-3/shBif1 cells were treated with serial dilution of ascorbate for 2, 6, and 24 h. Cell viability was measured and compared at 24 h. Data represent experiments carried out in triplicate (± SD).

necrosis [72]. However, excessive oxidative stress could subsequently damage the lysosome membrane and cause cell death, as those induced by ascorbate/menadione combination [23]. In our study, the autophagy inhibitor 3-MA decreased ascorbate-induced cell death, also, inhibited Bif-1 expression decreased the ascorbate-induced

cell death. It seems that, in this context, autophagy induced by ascorbate-generated ROS enhanced prostate cancer cell death. Both the peroxide-mediated and the autophagy-mediated mechanisms could potentially enhance cancer cell killing with high ascorbate concentrations. Taken together and combined with previous studies, the potential mechanisms of action of neoplastic cell death through H_2O_2 delivered by i.v. ascorbate are beginning to be elucidated and further studies are warranted.

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Conflicts of interest

There are no conflicts of interest.

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