ReLB Expression Determines the Differential Effects of Ascorbic Acid in Normal and Cancer Cells

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Abstract

Cancer cells typically experience higher oxidative stress than normal cells, such that elevating pro-oxidant levels can trigger cancer cell death. Although pre-exposure to mild oxidative agents will sensitize cancer cells to radiation, this pre-exposure may also activate the adaptive stress defense system in normal cells. Ascorbic acid is a prototype redox modulator that when infused intravenously appears to kill cancers without injury to normal tissues; however, the mechanisms involved remain elusive. In this study, we show how ascorbic acid kills cancer cells and sensitizes prostate cancer to radiation therapy while also conferring protection upon normal prostate epithelial cells against radiation-induced injury. We found that the NF-κB transcription factor ReLB is a pivotal determinant in the differential radiosensitization effects of ascorbic acid in prostate cancer cells and normal prostate epithelial cells. Mechanistically, high reactive oxygen species concentrations suppress ReLB in cancer cells. ReLB suppression decreases expression of the sirtuin SIRT3 and the powerful antioxidant MnSOD, which in turn increases oxidative and metabolic stresses in prostate cancer cells. In contrast, ascorbic acid enhances ReLB expression in normal cells, improving antioxidant and metabolic defenses against radiation injury. In addition to showing how ReLB mediates the differential effects of ascorbic acid on cancer and normal tissue radiosensitivities, our work also provides a proof of concept for the existence of redox modulators that can improve the efficacy of radiotherapy while protecting against normal tissue injury in cancer settings. Cancer Res; 77(6); 1345–56. ©2017 AACR.

Introduction

Prostate cancer is the most prevalent cancer in the United States and is the second leading cause of cancer deaths in men (1). The predominant prostate cancer therapy, ionizing radiation (IR), is used to treat more than 750,000 patients per year (2). Unfortunately, the therapeutic efficacy of IR tends to decrease when cancer cells develop adaptive responses to resist it. Even with modern conformal radiation therapy, biochemical failure occurs in approximately 45% of patients with a locally confined disease (3). In addition, corresponding with the wide-spread use of high-dose IR to treat prostate cancer, the incidence of radiation-related genitourinary toxicity has increased (4). Therefore, novel therapeutic strategies to enhance both radiosensitization in cancer cells and radioprotection in normal tissues are urgently needed.

Cancer cells are usually under higher oxidative stress than normal cells, and cellular redox state is thought to be important to cell fate. The level of oxidative stress is also critical to radiation response (5, 6). In addition to directly damaging DNA, IR can produce a large amount of free radicals that cause cell death (5). Because of water radiolysis, IR causes excess superoxide generation and allows leakage of electrons from the electron transport chain, resulting in mitochondrial dysfunction (7). However, IR also tends to induce adaptive reactive oxygen species (ROS) defense systems in cancers, which may lead to radioresistance (8). While increasing radiation intensity can improve the ability to control cancer growth, it presents the significant risks of increasing unwanted side effects, including injury to normal tissues and reduced quality of life for cancer survivors. Thus, an attractive radiation therapy would be one that exploits the intrinsic differences in the cellular redox statuses of normal cells and cancer cells by selectively boosting ROS generation in cancer cells to push them into oxidative stress overload while stimulating adaptive responses in normal cells.

Ascorbic acid, better known as vitamin C, has a somewhat controversial history as a therapeutic drug for cancer treatment (9, 10). Emerging studies suggest that only intraperitoneal or intravenous ascorbic acid, and not orally administered ascorbic acid, can reach pharmacological concentrations that kill cancer (10, 11). However, although clinical trials of ascorbic acid have
RelB in normal and cancer cells serves as a central regulator for acid on the modulation of cellular redox status. The expression of cancer cells account, in part, for the differential effects of ascorbic acid on the radioresistance of prostate cancer (17, 18). The present study examined the effects of ascorbic acid on cell survival and on the intrinsic differences in the cellular redox state of normal and cancer cells, in part, for the differential effects of ascorbic acid on the modulation of cellular redox status. The expression of RelB in normal and cancer cells serves as a central regulator for their opposing responses to radiotherapy.

Materials and Methods

Cell culture, cell transfection, and reagents

Human prostate cancer cell lines LNCaP and PC3, as well as normal human prostate epithelial viral transformed PZ-HPV-7 (PZ) cells, were obtained from ATCC. These cell lines are routinely checked for morphologic and growth changes to probe for cross-contaminated or genetically drifted cells. All cell lines used have been reauthenticated using the short tandem repeat (STR) profiling service by ATCC. Normal human epithelial cells (PrEC) were purchased from Lonza. The cells were cultured and maintained in the manufacturer’s suggested media. Plasmid-cloned RelB cDNA and siRNA for knocking down RelB were transfected into LNCaP and PC3 cells using a Lipofectamine 2000 kit (Life Technologies) according to the manufacturer’s instructions. Ascorbic acid (powder, USP/FCC grade, Fisher Chemical) was prepared as a 1 mol/L stock solution in sterile water, with sodium hydroxide added dropwise to adjust the pH to 7.0, as previously described. H2O2 (Sigma) solutions were prepared freshly prior to application to the cells.

Treatment and cell survival analysis

Cell survival rates were quantified by colony survival and MTT assays. For colony survival analyses, the cells were plated in 6-well plates at low densities and then treated with 0 to 1.0 mmol/L ascorbic acid for 2 hours, washed with fresh media, and incubated for 2 doubling times in the absence of ascorbic acid. Cell viability was detected by an MTT assay kit ( Trevigen) following the standard protocol. The IC50 for each cell line was calculated from a dose–response curve using GraphPad Prism 6.0 software (GraphPad software). For evaluations of combination therapy, PC3 and PZ cells were treated with ascorbic acid, IR, or a combination of the two. IR was performed at 1 hour after ascorbic acid treatment by a 250-kV X-ray machine (Faxitron X-ray Corp.) with peak energy of 120 kV, 0.05-mm Al filter, at a dose of 0 to 6 Gy. After a total 2-hour exposure to ascorbic acid, cells were incubated for 2 doubling times in the absence of ascorbic acid and assessed by MTT assay. Combination index (CI) values were calculated by CompuSyn 1.0 (CompuSyn), and the effects of combined IR and ascorbic acid treatment were evaluated according to the acknowledged range of CI as published (20).

Animals

Four- to 5-week-old male NCRNU (nu/nu athymic nude) mice were obtained from Taconic (Hudson). For formation of xenograft tumors, 1.8 × 106 cells mixed in Matrigel (BD Biosciences) were subcutaneously injected into the right flanks of the mice. Tumor volumes were routinely measured and their sizes calculated on the basis of a protocol described elsewhere (21). Animals with an average tumor size of 350 mm3 were randomized into 4 groups (n = 10) and treatment commenced with intraperitoneal injection as follows: (i) control, saline once daily; (ii) ascorbic acid, 4.5 g/kg once daily; (iii) IR, 2 Gy once every other day; and (iv) IR + ascorbic acid. The dosage of ascorbic acid was determined by conversion of clinical trial data and with reference to recent studies (22). For combination treatments, IR was performed at 1 hour after ascorbic acid injection. After treatment, the mice were observed daily and humanely killed when the tumor reached the maximum size of 1,500 mm3. The tumor, prostate, and bladder tissues were collected for protein and RNA analysis. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Lexington, KY). Approval Protocol No. 010777M2006.

Quantification of ROS level

An Amplex Red assay was used to quantify the levels of extracellular H2O2 after ascorbic acid and IR treatments. Briefly, cells were incubated with 50 μmol/L Amplex Red reagent (ThermoFisher) at 37°C for 10 minutes. Fluorescence was detected at ex/em 550/590 nm using a Gemini XPS Microplate Reader ( Molecular Devices). The extracellular H2O2 level was calculated by a standard curve and normalized to the cell number. MitoSox Red (ThermoFisher), a highly selective mitochondrial superoxide indicator for live cells, was used to estimate the levels of superoxide, and Mitotracker green FM (ThermoFisher) was used to locate mitochondria. Rotenone (200 nmol/L, Sigma), known to be a mitochondrial superoxide inducer, was used as a positive control. To account for superoxide-specific fluorescence, the cells were pretreated with 100 units/mL PEG-SOD (Sigma) for 24 hours followed by the treatment. In brief, cells were loaded with Mitotracker Green at 100 nmol/L for 15 minutes followed by 5 μmol/L Mitosox Red for 10 minutes at 37°C and rinsed 3 times with HBSS before measuring fluorescence. Cellular fluorescence intensity...
was detected using an Olympus IX71 fluorescence microscope and a Gemini XPS Microplate Reader at ex/em 510/580 nm.

Catalase and MnSOD treatments
To further determine the specific high ROS level induced by ascorbic acid and radiation, PC3 cells were treated with various forms of catalase or MnSOD, including catalase (100 U/mL), PEG-CAT (200 U/mL), PEG-MnSOD (100 U/mL), or adenovirus MnSOD. Catalase, PEG-catalase (PEG-CAT), and PEG-MnSOD were purchased from Sigma-Aldrich. For the adenovirus experiments, viral vectors utilized included AdCMVEmpty (AdEmpty) and AdCMV-MnSOD (AdMnSOD), manufactured by Viraquest, Inc. as previously described (23, 24). Approximately 10⁶ PC3 cells were plated in a 100-mm² tissue culture dish and incubated with the adenovirus constructs for 24 hours. Media were replaced with 5 mL of complete media for an additional 24 hours before cells were harvested for MTT assay or treated for ROS measurements.

Measurement of oxygen consumption rates, ATP, and lactate production
To determine whether ascorbic acid changes mitochondrial function in cancer and normal cells, a Seahorse Bioscience XF96 Extracellular Flux Analyzer was used to measure oxygen consumption rates (OCR) after 2 hours of treatment with 4 mmol/L ascorbic acid. The data were normalized with protein levels and expressed as the OCR in pmol/min/μg protein. Cellular ATP concentrations were measured using an ATP Assay Kit (Biomedical Research Service Center). Extracellular and intracellular lactate levels were measured by a Lactate Assay Kit (Biomedical Research Service Center). Data were normalized to the cell number, as indicated in each figure.

Western blots
Lysates from homogenized cells and tumor tissues were electrophoresed on a 10% to 12% (w/v) SDS-PAGE gel, transferred onto a nitrocellulose membrane, and subsequently incubated with primary antibodies against RelA, RelB, Bcl-xl, Bax, or GAPDH from Santa Cruz Biotech, or a DNase Fragment Assay Kit (ThermoFisher) and a Pierce Agarose ChIP Kit (ThermoFisher) was used to study RenB-mediated transcriptional regulation according to the manufacturer’s instructions. A potential RelB binding site was predicted in the promoter region of the human SIRT3 gene on the basis of a search of the Ensembl genome database and a recent study (25). Briefly, chromatin was pulled down using a RelB antibody (Santa Cruz Biotech), and a DNA fragment containing an NF-kB element located in the SIRT3 promoter region was analyzed by quantitative PCR (qPCR) with LightCycler 480 SYBR Green 1 Master Kit (Roche). PCR primer sequences for SIRT3 were 5'-gaattatgaaatgagcacag-3' (forward) and 5'-caggatcagcaagctg-3' (reverse). Rabbit IgG antibody was used as a negative control. ChIP-qPCR data were normalized by input preparation.

Intracellular catalase, Gpx and MnSOD enzymatic assay
The activities of catalase and Gpx were measured by a Catalase- and Gpx-Activity Assay Kit (Abcam) and a Gpx Cellular activity assay Kit (Sigma) according to the manufacturers’ protocols, respectively. MnSOD activities were measured by the nitroblue tetrazolium-bathocuproin sulfonate reduction inhibition method. Sodium cyanide (2 mmol/L) was used to inhibit Cu/ZnSOD activity as a previous study described (26).

Quantitative and statistical data analyses
Multiple independent experiments were conducted for each set of data presented. Image data were quantified using the quantitative imaging software Image-pro Plus 6.0 (Media Cybernetics). Toxicity comparisons of multiple groups were analyzed using ANOVA and a post-hoc test. Data represent the mean ± SEM. Kaplan–Meier survival curves, and the log-rank test were performed for comparison of the survival curves in animal experiments. Statistical significances of other experiments were analyzed using one-way ANOVA and Tukey multiple comparison tests. All analyses were performed with IBM SPSS 21.0 software (Microsoft). Differences with an associated P < 0.05 were considered to be significant.

Results
Ascorbic acid enhances radiosensitivity in prostate cancer cells but protects normal cells from radiotoxicity
To determine the cytotoxicity of ascorbic acid in prostate cancer and normal cells, LNCaP, PC3, PrEC, and PZ cells were plated for colony survival assays and MTT assays. As shown in Fig. 1A and B, high doses of ascorbic acid alone efficiently killed cancer cells but exerted no or minimal effect on normal

Ascorbic Acid Exhibits Opposing Effects in Cancer and Normal Cells

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Interestingly, ascorbic acid appears to be more efficient in killing aggressive prostate cancer PC3 cells than LNCaP cells. On the basis of a dose–effect curve, the IC50 values for PC3, LNCaP, PrEC, and PZ cell lines were quantified as 3.96, 12.81, 36.56, and 33.79 mmol/L, respectively, indicating that ascorbic acid has different cytotoxic effects on prostate cancer and normal cells.

To determine the capacity of ascorbic acid to sensitize prostate cancer cells to IR, we used the protocol for radiosensitization of PC3 cells described in our previous study (27), taking into consideration the relative ascorbic acid IC50 value in clinical application (28). The total dose of ascorbic acid was kept at 2 mmol/L (for 1/2 IC50) or 4 mmol/L (for the full IC50). Pretreatment with ascorbic acid significantly increased the radiosensitivity of PC3 cells in a dose-dependent manner within an IR range of 0.5 to 6 Gy (Fig. 1C). Interestingly, pretreatment with ascorbic acid resulted in the opposite effect in PZ cells, indicating that ascorbic acid actually protects normal cells against the cytotoxicity induced by IR (Fig. 1C). To assess the combined effects of ascorbic acid and IR, the CI value for each dose was calculated by CompuSyn software on the basis of the Chou–Talalay method (20). A dose of 4 mmol/L ascorbic acid conformed to the CI range description in that it displayed a synergistic effect on IR from 1 to 6 Gy in PC3 cells (Supplementary Table S1). On the contrary, both doses of ascorbic acid in PZ cells displayed antagonistic effects on IR over a dose range of 0.5 to 6 Gy. These results suggest that equivalent pharmacological doses of AA can exert different radiosensitization effects in prostate cancer and normal cells.

Ascorbic acid differentially modulates cellular ROS levels in cancer and normal cells

To determine the effect of ascorbic acid on extracellular ROS levels in cancer and normal cells, the levels of ROS and mitochondrial superoxide anion were measured with and without ascorbic acid treatment. As shown in Fig. 2A, the basal level of extracellular H2O2 was slightly but significantly higher in prostate cancer cells than in normal cells. In comparison to the result in PZ cells, ascorbic acid treatment induced a significant increase in ROS in prostate cancer cells, especially PC3 cells (Fig. 2A). Pretreatment with PEG-CAT or catalase obviously inhibited such extracellular H2O2 increases and weakened toxicity of ascorbic acid in PC3 cells (Supplementary Figure S1).
Fig. S1A and S1B). These results are consistent with previous reports that ascorbic acid produces H$_2$O$_2$ by oxidative reaction with metal ions in extracellular fluid and exerts cytotoxic effects (14, 15).

To evaluate intracellular redox status, superoxide levels in mitochondria, the primary source of cellular ROS, were quantified in cancer and normal cells. Mitotracker green staining with negligible stimulated ROS was used to normalize MitoSox red to quantify mitochondrial superoxide anion induced by ascorbic acid with rotenone as a positive control. Ascorbic acid induced superoxide generation in PC3 cells, but the effect was mitigated by adding PEG-SOD (Supplementary Fig. S1C). Consistent with the results for cytotoxicity in Fig. 1, ascorbic acid increased IR-induced superoxide generation in PC3 cells but decreased it in PZ cells (Fig. 2B). Increasing endogenous MnSOD expression in PC3 cells clearly decreased superoxide, especially IR-induced superoxide generation after ascorbic acid treatment (Supplementary Fig. S1D). These results suggest that ascorbic acid amplifies IR-induced cellular ROS level in cancer cells. They also suggest that ascorbic acid suppresses IR-induced ROS generation in normal cells.

Ascorbic acid differentially modulates mitochondrial function in cancer and normal cells

To test whether altering cellular redox status is associated with mitochondrial function, the OCR in the ascorbic acid–treated cells was measured using a Seahorse Bioscience XF96 OxygenFlux Analyzer. As shown in Fig. 3A, ascorbic acid treatment decreased basal, ATP-linked, and maximal OCR but not reserve capacity in PC3 cells. In contrast, ascorbic acid treatment increased the maximal OCR and reserve capacity of PZ cells. To test whether such effects modulate the energy production of cells, the cellular ATP and lactate levels were determined with and without treatment. As expected, pretreatment with ascorbic acid significantly diminished intracellular ATP when combined with IR, but increased both extracellular and intracellular lactate production in PC3 cells (Fig. 3B–D). In contrast, combined ascorbic acid and IR treatment increased ATP production but decreased lactate concentrations in normal PZ cells (Fig. 3B–D). These results suggest that ascorbic acid–mediated alterations in cellular oxidative and metabolic stresses play pivotal roles in the radiation response of both prostate cancer and normal cells. Importantly, ascorbic acid exacerbates mitochondrial dysfunction in cancer cells but alleviates radiation-induced mitochondrial dysfunction in normal cells.

Ascorbic acid inversely regulates RelB expression in cancer and normal cells

NF-kB signaling is involved in multiple biologic processes responsive to ROS (29). The best known activator of NF-kB is RelA, which is associated with radioresistance in many types of cancer. Our previous findings demonstrate that RelB is also highly expressed in prostate cancer cells and is a major contributor to radioresistance (18, 30). To probe which member of...
the NF-κB family is affected by ascorbic acid, we first determined whether ascorbic acid regulates the expression of RelA and RelB in prostate cancer and normal cells at the mRNA and protein levels. While ascorbic acid slightly increased RelA protein levels in both cancer and normal cells, ascorbic acid strongly downregulated RelB protein and mRNA in cancer cells and upregulated RelB in normal cells (Fig. 4A). To further verify this finding, an NF-κB binding assay was performed to confirm ascorbic acid activation or suppression of RelA and RelB. As shown in Fig. 4B, RelB binding activity was strongly suppressed in PC3 cells but apparently activated in PZ cells after ascorbic acid treatment alone. Importantly, pretreatment with ascorbic acid significantly diminished IR-induced RelB activation in PC3 cells. No significant effect of ascorbic acid was observed on RelA binding activity, but a small but significant increase was observed with combination treatment in PZ cells.

To further confirm the transcription effect of RelB on its target genes, the NF-κB–regulated genes Bcl-xL and Bax were quantified to verify the regulation of the NF-κB pathway by ascorbic acid in prostate cancer and normal cells. Consistently, ascorbic acid downregulated Bcl-xL but upregulated Bax in PC3 cells, whereas the reverse effect of ascorbic acid was observed in PZ cells (Supplementary Fig. S2A). Finally, to confirm the role of RelB in ascorbic acid–induced cell killing of prostate cancer cells, the level of RelB was manipulated by either overexpression of RelB in LNCaP cells or knockdown of RelB in PC3 cells. Ascorbic acid–mediated cytotoxicity was increased in RelB-silenced PC3 cells but reduced in RelB-overexpressed LNCaP cells (Fig. 4C, Supplementary Fig. S2A).

RelB acts as a central regulator in response to oxidative and metabolic stresses in cancer cells

Generally, the radiosensitization we find in cancer cells is due to the oxidative and metabolic stresses induced by ascorbic acid. Because our data showed that RelB plays an important role in ascorbic acid–induced cell killing of prostate cancer cells, it was necessary to verify the functions of RelB in oxidative and metabolic stresses. As shown in Fig. 5A, knockdown of RelB reduced basal ATP production and increased intracellular lactate level. When treated with ascorbic acid, IR, or combination treatment, RelB-silenced PC3 cells displayed more aggravated metabolic stress compared with the control group (Fig. 5A). The OCR data further confirmed the effect of suppressing RelB on mitochondrial function in cancer cells, especially when treated with ascorbic acid and IR (Fig. 5B). To confirm the role of RelB in response to oxidative stress, the activities of ROS-related enzymes were evaluated in RelB-silenced PC3 cells. As shown in Fig. 5C and D, knocking down RelB markedly weakened the ability of ROS elimination in cancer cells, which was revealed by the reduction of catalase, GPX, and MnSOD activities. These results confirm the role of RelB in oxidative and metabolic regulations. Furthermore, they confirm that RelB may be a central regulator that adjudicates the differential effects of ascorbic acid in normal and cancer cells.
RelB transcriptionally regulates SIRT3 in response to ascorbic acid treatment

SIRT3, a member of the sirtuin family of NAD$^+$-dependent protein deacetylases, is known to play a critical role in maintaining mitochondrial function, ROS response, and cell proliferation, as well as in inducing radioresistance in cancers. Recently, a sequential action of SIRT1–RelB–SIRT3 has been reported in sepsis (31), but the related mechanisms remain to be fully elucidated. To probe the relationship of SIRT3 with RelB in ascorbic acid–induced cytotoxicity in cancer cells and the protective effect in normal cells, the levels of SIRT1 and SIRT3 relative to RelB were quantified in PC3 and PZ cells. As shown in Fig. 6A, upregulation and downregulation of SIRT3 correlated with RelB in the ascorbic acid–treated cells, but no significant changes were observed in SIRT1. Interestingly, MnSOD, a typical NF-$\kappa$B–regulated mitochondrial antioxidant enzyme, was suppressed in PC3 cells but enhanced in PZ cells treated with ascorbic acid (Fig. 6A).

Manipulation of RelB expression in cancer cells altered SIRT3 levels, indicating that SIRT3 is regulated by RelB (Fig. 6B). Subsequently, RT-PCR showed that RelB transcriptionally regulated SIRT3 in the ascorbic acid–treated cells (Fig. 6C). Furthermore, chromatin was pulled down by a RelB antibody, and a promoter region of the human SIRT3 gene containing an NF-$\kappa$B element was quantified by qPCR (Fig. 6D). The amount of the pulled down promoter fragment was reduced by ascorbic acid treatment in PC3 cells but increased in ascorbic acid–treated PZ cells. When combined with IR, ascorbic acid...
Figure 5.
The regulation of RelB on redox and metabolic homeostasis. A, Intracellular ATP levels, extracellular and intracellular lactate levels were measured after treatment with ascorbic acid and IR at the indicated doses in PC3 and in RelB-silenced PC3 cells. B, After ascorbic acid and IR treatment, OCR in PC3 and in RelB-silenced PC3 cells was measured by a Seahorse Bioscience XF96 OxygenFlux Analyzer. C, Level of cellular ROS was estimated by the ratio of H2DCFDA to Carboxy-DCFDA. PEG-CAT was used as a control to remove ROS generated by ascorbic acid. D, Catalase activity, Gpx activity, and MnSOD activity were measured after treatment with ascorbic acid and IR in PC3 and in RelB-silenced PC3 cells. Two-tailed Student t test was performed for comparisons of RelB-silenced group to control group.
consistently suppressed the activated RelB–SIRT3 signal in cancer cells but further activated the RelB–SIRT3 signal in normal cells.

Ascorbic acid enhances radiosensitivity of prostate cancer in vivo

To further confirm our findings in vitro, a tumor-bearing mouse model was used to verify the effect of ascorbic acid on tumor growth. Mice were subcutaneously injected with PC3 cells followed by ascorbic acid and IR treatments. Mice were humanely killed and tumors as well as prostate and bladder tissues were collected when the tumor volume reached 1,500 mm³. The tumor growth rate of each group shown in Fig. 7A demonstrates the efficacy of ascorbic acid in sensitizing prostate cancer to radiation therapy. There was a trend toward significance in the differences between ascorbic acid–treated and untreated mice. The time needed for the tumors in each group to reach 1,500 mm³ was independently analyzed (Supplementary Fig. S3A).

mRNA and protein levels of RelB and its targets, SIRT3 and MnSOD, were quantified in the extracted tumor tissues. Consistent with the results obtained in vitro, IR increased the levels of RelB, SIRT3, and MnSOD, but ascorbic acid eliminated the increases (Fig. 7B). In addition, prostate and bladder tissues were used to probe whether ascorbic acid activates a protective response against radiation injury in normal tissues. Consistent with the results obtained in vitro, IR increased the levels of RelB and SIRT3 mRNA in normal prostate and bladder tissues (Fig. 7C).

Overall, ascorbic acid enhances the radiosensitivity of prostate cancer cells but protects normal cells from radiotoxicity through RelB-dependent transcriptional regulation, with consequences for downstream target genes, such as SIRT3 and MnSOD, that lead to distinct responses to radiation, as illustrated in a working model based on the results obtained to date (Fig. 7D).

Discussion

The problems of the radioresistance of cancer tissues and the toxic side effects of IR in normal tissues have been extensively investigated in both scientific and clinical settings. However, traditional approaches have focused on ensuring that protecting normal tissues from injury does not also reduce the therapeutic efficacy of radiation. Here, we demonstrate that ascorbic acid, a redox active agent, can enhance the therapeutic efficacy of radiation therapy while simultaneously protecting normal tissues against the side effects of radiation therapy.

Ascorbic acid enhances traditional radiotherapy and chemotherapy of cancer has been reported since 1977 (14, 15, 32–35). In the intervening decades, ascorbic acid has been used to alleviate some of the side effects of radiation therapy during cancer treatment (34, 36). A recent study by Du and colleagues clearly shows the radiosensitization induced by pharmacologic ascorbic acid in pancreatic cancer cells (15). These authors also observed that ascorbic acid also potentially protects the gastrointestinal tract from IR in vivo. Although it was not a major focus of the study, the observation by Du and colleagues is consistent with our finding that ascorbic acid indeed protects normal prostate and bladder tissues from IR.
Elevated ROS levels, which are essential for tumorigenesis and metastasis, have been observed in many types of cancer. High levels of ROS may reveal a specific vulnerability of malignancy that can be used to selectively enhance cell death by further increasing the level of cellular ROS. Here, we show that ascorbic acid acts as a pro-oxidant at pharmacologic doses and differentially modulates cellular responses to ROS in normal and cancer cells. Our results are consistent with previous studies demonstrating that the cytotoxicity induced by ascorbic acid is primarily mediated by \( \text{H}_2\text{O}_2 \) \(^{14, 15, 19 \text{ and } 22} \). However, the previous studies did not take into consideration the respective distinct basal redox state of normal and cancer cells. Ascorbic acid sensitizes cancer cells to radiation by downregulating RelB-SIRT3 signal, which in turn aggravates oxidative and metabolic stresses. In contrast, in normal prostate epithelial cells, \( \text{H}_2\text{O}_2 \) generated from the redox reaction of ascorbic acid upregulates RelB, leading to increased SIRT3 levels, which enhances cellular stress defense systems. In animal experiments, other data were analyzed using one-way ANOVA with post-hoc Tukey honest significant difference test.
acid, leading to the observed opposing radiation responses in prostate cancer and normal cells. Repression of IR-induced RelB activation in cancer cells results in diminished oxidative defense capacity and subsequently enhances radiosensitivity through mitochondrial dysfunction. On the contrary, upregulation of RelB serves as a major mechanism by which ascorbic acid protects normal tissues against radiotoxicity, through upregulation of antioxidant enzymes and the mitochondrial function of scavenging ROS. RelA has been reported to be involved in RelB transcriptional activation (39); further studies will be needed to determine whether and how RelA participates in a meaningful change of RelB as it relates to ascorbic acid.

Recently, metabolic alterations in cancer cells due to ascorbic acid–induced oxidative stress have been the subject of intense investigation (15, 40). Although a high rate of aerobic glycolysis in tumors, known as the Warburg effect, has been observed in various types of cancer, cancers have functional mitochondria, and mitochondrial respiration is necessary for cancer cell proliferation and resistance to therapy (41, 42). The present study shows that the mitochondria in prostate cancer cells become dysfunctional, with downregulated MnSOD, after ascorbic acid treatment. As a MnSOD transcriptional regulator, RelB modulates dysfunctional, with downregulated MnSOD, after ascorbic acid shows that the mitochondria in prostate cancer cells become dysfunctional, with downregulated MnSOD, after ascorbic acid.

Sirtuins are NAD+-dependent histone deacetylases in mammalian cells and are involved in an array of critical cellular functions (43–46). Of the 7 human sirtuins, SIRT3 is the best characterized in its regulation of many aspects of mitochondrial function: Physiologically, SIRT3 interacts with subunits of complexes I and II of the electron transport chain to improve mitochondrial respiration (47). SIRT3 also deacetylates and activates MnSOD to maintain the antioxidant defense system in cells (48). A recent study described the expression of SIRT3 as a sequential action of the SIRT1/RelB axis in a sepsis model (31). The present study suggests that the RelB/SIRT3 signaling axis may play a critical role in ascorbic acid treatment independent of SIRT1 levels. Our results demonstrate that RelB regulates SIRT3 expression through binding to its promoter region. Repression of RelB-activated SIRT3 transcription by ascorbic acid aggravates metabolic stress in cancer cells. In contrast, upregulation of SIRT3 improves the ability of mitochondria to defend against metabolic stress in normal cells. These results suggest that RelB may be a unique target for treatment of radiation-resistant prostate cancer.

Considering the unsatisfactory results of clinical trials of high doses of oral ascorbic acid, and the reported successes of high doses of intravenous ascorbate (13, 49), the complexity of the mechanisms involved in ascorbic acid treatment deserves further investigation. The present study indicates a promising anticancer effect of ascorbic acid that is dependent on cell properties such as the basal redox state of the cancer and normal cells. The present study also reveals cell-dependent ROS generation in ascorbic acid treatment and identifies the RelB/SIRT3/MnSOD axis as a critical contributor to ascorbic acid–induced radiosensitization of cancer cells and radioprotection of normal cells (Fig. 7D). Thus, while additional mechanistic studies are needed to fully understand the biologic function of ascorbic acid, we anticipate that other redox-based anticancer therapeutics with protective properties against cytotoxic therapy will be discovered and that they will have a significant impact on the care of patients with cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Development of methodology: X. Wei, Y. Xu, F.F. Xu, D. Schnell
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