Ascorbate (vitamin C) induces cell death through the apoptosis-inducing factor in human breast cancer cells

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Abstract. Although ascorbate (Vitamin C) has been shown to inhibit cell growth and induce cell death in variety of cancer cells, results reported in other studies are inconsistent with this conclusion. It was previously reported that ascorbate induces apoptosis in human breast cancer cells. However, the molecular mechanism for this is not clear. In this study, we demonstrate that ascorbate induces cell death through the apoptosis-inducing factor (AIF) in the human breast cancer cell lines, SK-BR3 and Hs578T, but not in a normal breast cell line, Hs578. Ascorbate treatment caused the nuclear translocation of AIF, which is retained in the mitochondria in healthy cells, but caspase cleavage is not induced. Moreover, MG132, an inhibitor of AIF release from mitochondria, blocked the induction of cell death. Furthermore, cells that had been treated with human AIF-specific siRNA resisted cell death induced by ascorbate, implying that the translocation of AIF from mitochondria to the nucleus is responsible for ascorbate-mediated cell death. Therefore, these results suggest that ascorbate activates a caspase-independent and AIF-mediated cell death pathway in human breast cancer cells, SK-BR3, and Hs578T.

Introduction

The effect of ascorbate (vitamin C) in cancer treatment remains controversial (1). It was previously reported that ascorbate induces cell cycle arrest and apoptosis in various tumor cells, such as lymphoma cells and leukemia cells (2-4), melanoma cells (5), brain tumor cells (6,7), prostate cancer cells (8,9), and stomach cancer cells (10). However, the molecular mechanism of ascorbate as an anti-tumor effector has not been fully elucidated.

Apoptosis is a tightly programmed form of cell death in which cells actively participate in their own destruction. Apoptosis is mainly triggered by either the extrinsic pathway (11), which is also known as the death receptor pathway, or by the intrinsic pathway (12), which may affect cellular organelles, including the nucleus, the endoplasmic reticulum (ER), lysosomes or mitochondria (13). Both the extrinsic and the intrinsic pathways of apoptosis lead to cell shrinkage, chromatin condensation, nuclear fragmentation, blebbing and phosphatidylserine exposure on the surface of the plasma membrane (14).

Apoptotic cell death largely proceeds in either a caspase-dependent or caspase-independent manner (15). It has been discovered that, in response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effectors such as the apoptosis-inducing factor (AIF) (15-17). AIF, when released from mitochondria, is translocated to the nucleus where it triggers chromatin condensation and large DNA fragmentation in a caspase-independent manner, indicating that it is an important factor in cell death in MCF-7 cells with depleted caspase 3 or 8 (18).

In this study, we examined the anti-cancer effect of ascorbate using human breast cancer cells. We showed that ascorbate treatment induced apoptosis through the nuclear translocation of AIF. We also found that ascorbate-induced apoptosis was not continuous with Ca2+ signaling and caspase-dependent apoptosis, using pharmaceutical inhibitors, such as nifedipine, a Ca2+ inhibitor, and a pan-caspase inhibitor. We report that human breast tumor-derived cell lines are sensitive to ascorbate-induced cell death and discuss the potential mechanism involved in this process.

Materials and methods

Cell cultures, materials and siRNA transfection. Hs578T and SK-BR3, human breast carcinoma cells, and Hs578, human...
breast normal cells were maintained in DMEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY) and penicillin-streptomycin (50 U/ml). Nifedipine as Ca\(^{2+}\) blocker, pan-caspase inhibitor, and MG132 as blocker of AIF release (19) were purchased from Sigma Chemical Company. Cells transfected with the siRNA of human AIF (5’GAUCCU CCCCGAUAUACCUTT-3’) were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Scrambled RNA as control was obtained from Proligo LLC (Boulder, CO, USA).

**Cell cytotoxicity.** Cell viability was determined by trypan blue exclusion, by counting at least 500 cells in each culture. Cells were treated with 0.1, 0.2, 0.5, and 1 mM of ascorbate (Sigma, St. Louis, MO) for 24 h, and then live and dead cells were counted using the trypan blue exclusion assay.

**Immunoblot analysis.** Cells lysates were prepared with RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 μM EGTA, 1% Triton X-100, 50 mM NaF, 10 mM Na_3PO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM DTT). Protein extracts were normalized for concentration using the Bradford assay and 20 μg of total cell protein per sample was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PolyScreen membrane (NEN, Boston, MA). The membranes were blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and probed with one of the following antibodies: anti-cleaved caspase 9, anti-cleaved caspase-3, antibodies (Cell Signaling Technology, Beverly, MA); anti-tubulin, AIF, and Hsp60 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were detected with either goat anti-mouse, goat anti-rabbit, or donkey anti-goat horseradish peroxidase-conjugated secondary antibody by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

**Preparation of mitochondrial extracts.** Cells were resuspended in an isotonic buffer containing 10 mM HEPES pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and homogenized using a 26-gauge syringe needle. Cell homogenates were spun at 1,000 g to remove unbroken cells, nuclei, and heavy membranes. The supernatant was then spun again at 14,000 g for 30 min to yield the mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondrial fraction was washed once with extraction buffer, and finally resuspended in RIPA buffer for Western blot analysis.

**Immunofluorescence analyses.** For AIF staining, cells were fixed with PBS/3.7% paraformaldehyde at RT for 15 min, and then permeabilized in PBS/0.1% Triton X-100.1 mg/ml RNase A at 37°C for 30 min. The permeabilized cells were treated with 0.5 μg/ml of NaBH₄ so that autofluorescence was reduced. The cells were then immunostained by the anti-AIF goat polyclonal antibody (Santa Cruz Biotechnology) at 1:100 dilution, and then by Mitotracker Orange CMTMRos (Molecular Probes, Eugene, OR), and DAPI. After washing 3 times with PBS, coverslips were mounted on microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by an Olympus DP50 digital camera (Olympus Optical, Japan).

**Results**

Ascorbate can induce cell death in human breast cancer cells. We examined cell viability in human breast cancer cells, Hs578T and SKBR3 and normal cells, Hs578 during treatment with various doses of ascorbate. Cancer cells treated with ascorbate induced cell death after 24 h, whereas normal cells...
were not affected (Fig. 1). The cell death-inducible dose of ascorbate in Hs578T and SKBR3 was lower in other cancer cells, implying that sensitivity to cell death by ascorbate in both cells is higher. In addition, normal breast cells, Hs578, were derived from a patient who also produced Hs578T, breast cancer cells. In normal cells and cancer cells derived from the patient, ascorbate induced cell death was observed only in breast cancer cells, Hs578T, but no cytotoxicity was found in the case of normal breast cells, Hs578, suggesting that ascorbate could be used as an anti-tumor agent without any significant side-effects.

Ascorbate induces caspase-independent cell death through the nuclear translocation of the apoptosis inducing factor. To investigate how ascorbate induces cell death, we examined key regulators in ascorbate-induced cell death after various treatments with inhibitors, such as nifedipine, a Ca\(^{2+}\) blocker, a pan-caspase inhibitor, and MG132 as a blocker of AIF release (19), and observed whether cell death induced by ascorbate was suppressed by these various inhibitors. It was previously reported that ascorbate induces apoptosis via Ca\(^{2+}\) signals of the endoplasmic reticulum (ER), which regulates protein synthesis, protein folding and trafficking, intracellular Ca\(^{2+}\) levels (20), and participates in the initiation of cell death signaling (21). We found that the blockage of the Ca\(^{2+}\) signal by nifedipine did not suppress ascorbate-induced cell death, and apoptosis was also not inhibited by pan-caspase inhibitor in human breast cancer cells, Hs578T and SKBR3 (Fig. 2). However, ascorbate-induced cell death was suppressed after MG132 treatment, which blocks AIF release (19) (Fig. 2). We therefore examined the activities of caspase 3 and 9, but neither were activated upon ascorbate treatment (data not shown). Therefore, these results imply that ascorbate induces cell death through the nuclear translocation of AIF without activation of either of the caspases.

To confirm the translocation of AIF from mitochondria to the nucleus upon ascorbate treatment, we carried out immunostaining for AIF (Fig. 3). Co-staining with Mitotracker Orange CMTMRos to label AIF (red) revealed that the punctate nucleic DAPI staining (blue) colocalizes with AIF, yielding a baby blue composite signal when DAPI and Mitotracker images of AIF were overlaid. The immunostaining experiments showed that AIF proteins were mainly localized in the mitochondria. Further immunostaining showed that AIF became localized in nuclei only after ascorbate treatment (Fig. 3).

We next examined the effects of AIF silencing using small interfering RNA (siRNA). Cell fractionation showed that AIF protein was weakly detected in the mitochondrial fraction, but strongly in the cytosol in ascorbate-treated cells, while cytosolic AIF protein levels after ascorbate treatment were decreased in AIF-siRNA-treated cells as compared with that of cells treated with scrambled RNA, equivalent to the cells that had been treated with MG132 (Fig. 4A). In parallel with AIF silencing, the populations of dead cells were decreased in the presence of ascorbate (Fig. 4B), implying that AIF silencing renders cells resistant to ascorbate. These results
suggest that the nuclear translocation AIF is essential for ascorbate-induced cell death in human breast cancer cells and that ascorbate could be a potential anti-tumor agent.

Discussion

The findings herein show that ascorbate induces cell death through the release of AIF from mitochondria in human breast cancer cells in a caspase-independent manner. However, normal breast cells, Hs578, which were derived from the same patient who was the source of Hs578T, breast cancer cells, were insensitive to vitamin C.

The use of ascorbate for breast cancer therapy should be viewed with some caution because of an overall lack of clear research findings. Recent studies reported that a mixture of ascorbate and other compounds also induces cell cycle arrest or cell death only in human breast cancer cells. For example, breast cancer cell proliferation was inhibited by extracts from cultured strawberries (22), a cocktail of ascorbate and copper (23), a mixture of retinoic acid and ascorbate (24), and a mixture of lysine, proline, ascorbate and green tea extract was reported to have antitumor activity (25), whereas other groups reported that ascorbate inhibits tumor angiogenesis through the suppression of HIF-1α and HIF transcriptional targets (26).

Ascorbate induced cell death in human breast cancer cells, Hs578T and SK-BR-3, while failed to induce cell death in human normal breast cells, Hs578. Therefore, we analyzed molecular mechanisms by which ascorbate induces cell death (Fig. 1). It has previously been reported that ascorbate induces cell death via PKCδ pathway in neuroblastoma cells (27), the Ca²⁺ signal in human hepatoma cells (28), glioblastoma cells, renal carcinoma cells (29), and the caspase-dependent pathway in melanoma cells (30). Based on these studies, we examined the effect of some chemical inhibitors, such as rottlerin, a PKCδ specific inhibitor, nifedipine, a Ca²⁺ signal blocker, a pan-caspase inhibitor, and MG132, an AIF blocker (19), on ascorbate-induced cell death. Rottlerin, nifedipine, and pan-caspase inhibitor (data not shown) had no effect on ascorbate-induced cell death. However, MG132 suppressed the cell death induced by ascorbate (Fig. 2). These results suggest that ascorbate induces cell death through a caspase-independent pathway in human breast cancer cells. The release of AIF from mitochondria by ascorbate treatment was clearly found from mitochondria by ascorbate treatment was clearly found by immunostaining using AIF-specific antibody, and the inhibitory effect of MG132 on AIF release was also confirmed (Fig. 3).

We next examined the effects of AIF silencing using interfering RNA (siRNA) (31). Endogenous AIF protein levels were decreased in AIF-siRNA-treated cells, but not in ascorbate-treated cells (Fig. 4A). In parallel with AIF silencing, the populations of dead cells were decreased in the presence of ascorbate (Fig. 4B), implying that AIF is necessary for cell death induced by ascorbate in human breast cancer cells.

In this study, we demonstrate that ascorbate induces cell death in a caspase-independent manner through AIF release from mitochondria in human breast cancer cells. Biochemical analyses showed that ascorbate induces AIF release from mitochondria, thereby inducing cell death. However, MG132 as a blocker of AIF release and AIF-SiRNA failed to completely suppress cell death induced by ascorbate, suggesting that there may be another pathway by which ascorbate induces cell death, in addition to the AIF signal. Collectively, these findings suggest that ascorbate release from mitochondria is critical for inducing cell death in cells damaged by ascorbate.

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References


