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ORIGINAL ARTICLE

SVCT-2 in breast cancer acts as an indicator for L-ascorbate treatment

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L-ascorbate (L-ascorbic acid, vitamin C) clearly has an inhibitory effect on cancer cells. However, the mechanism underlying differential sensitivity of cancer cells from same tissue to L-ascorbate is yet to be clarified. Here, we demonstrate that L-ascorbate has a selective killing effect, which is influenced by sodium-dependent vitamin C transporter 2 (SVCT-2) in human breast cancer cells. Treatment of human breast cancer cells with L-ascorbate differentially induced cell death, dependent on the SVCT-2 protein level. Moreover, knockdown of endogenous SVCT-2 via RNA interference in breast cancer cells expressing high levels of the protein induced resistance to L-ascorbate treatment, whereas transfection with SVCT-2 expression plasmids led to enhanced L-ascorbate chemosensitivity. Surprisingly, tumor regression by L-ascorbate administration in mice bearing tumor cell xenograft also corresponded to the SVCT-2 protein level. Interestingly, SVCT-2 expression was absent or weak in normal tissues, but strongly detected in tumor samples obtained from breast cancer patients. In addition, enhanced chemosensitivity to L-ascorbate occurred as a result of caspase-independent autophagy, which was mediated by beclin-1 and LC3 II. In addition, treatment with N-acetyl-L-cysteine, a reactive oxygen species (ROS) scavenger, suppressed the induction of beclin-1 and LC3 II, implying that the differential SVCT-2 protein-dependent L-ascorbate uptake was attributable to intracellular ROS induced by L-ascorbate, subsequently leading to autophagy. These results suggest that functional SVCT-2 sensitizes breast cancer cells to autophagic damage by increasing the L-ascorbate concentration and intracellular ROS production and furthermore, SVCT-2 in breast cancer may act as an indicator for commencing L-ascorbate treatment.

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INTRODUCTION

L-ascorbate (L-L-ascorbic acid, vitamin C) is a necessary nutrient employed as a cofactor by numerous enzymes related to metabolism.[1–3] The efficacy of L-ascorbate in cancer treatment currently remains a subject of controversy.[4] Several reports have been published on the beneficial effects of L-ascorbate in anticancer therapies, showing that L-ascorbate has inhibitory effects on various cancer cells, including melanoma, brain tumor, prostate cancer and stomach cancer,[5–8] and administration of daily doses of 10 g L-ascorbate has a positive effect on cancer treatments.[9–11] In contrast, other studies report that similar doses of L-ascorbate have no effects on cancer patient survival.[12,13] Two contradictory reports require further investigation of the effects of L-ascorbate on cancer, specifically, in terms of (1) the differences in chemosensitivity of various cancer cells to L-ascorbate, and (2) detailed mechanisms underlying inhibitory action of L-ascorbate against cancer cells.

L-ascorbic acid is transported into cells by sodium-dependent vitamin C transporters (SVCTs) as the reduced form of vitamin C.[14] The two isoforms of SVCT have been cloned and characterized in rats[14] and humans,[15] and display distinct tissue distribution. Specifically, hSVCT-1 is detected in the epithelial tissue and liver[14,16] whereas hSVCT-2 is expressed in most tissues, with the exception of the lung and muscle.[17] Moreover, hSVCT-2 exhibits higher affinity for L-ascorbic acid than hSVCT-1.[18] Recent interesting studies show that sodium-dependent vitamin C transporter 2 (SVCT-2) is the key protein responsible for L-ascorbate uptake in the liver,[19] and genetic variations in SVCT-2 modify the risk of HPV16-associated head-and-neck cancer.[20] These findings suggest that SVCT-2, the main functional protein among the two isoforms, is associated with cancer.

In this investigation, we propose the hypothesis that differential chemosensitivity of various cancer cells from the same tissue to L-ascorbate is related to the extent of SVCT-2 expression that is mainly responsible for L-ascorbate uptake. Moreover, we investigate the mechanisms underlying L-ascorbate action on cancer cells. To our knowledge, this is the first report demonstrating a relationship between SVCT-2 and chemosensitivity of breast cancer cells to L-ascorbate treatment.

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RESULTS

Human breast cancer cells display different chemosensitivities to ascorbate by the level of SVCT-2 protein

To determine the effects of L-ascorbate on cell viability, we treated multiple human breast cancer and normal mammary gland epithelial cells (HMEC) with various doses of L-ascorbate. The viability of most breast cancer cells was decreased, with the exception of normal mammary gland cells (Figure 1a). Interestingly, we observed differences in chemosensitivity to L-ascorbate among the breast cancer cell lines examined. BT20 and MDA-MB231 cells, which showed low expression of SVCT-2 protein, exhibited high resistance to L-ascorbate, whereas T47D, Hs578T and SKBR-3 cells, which showed high expression of SVCT-2, were most sensitive to L-ascorbate (Figure 1a). To ascertain whether the difference in chemosensitivity to L-ascorbate is a result of different concentrations of L-ascorbate flow into cancer cells, we initially assayed the expression of SVCT-2, which is responsible for L-ascorbate uptake into cells, in a variety of human breast cancer cells (Figure 1b). Surprisingly, the SVCT-2 protein level was inversely correlated with cell viability (Figure 1), implying that differential sensitivity to L-ascorbate is related to SVCT-2 protein expression. Accordingly, we further analyzed whether the difference in chemosensitivity to L-ascorbate is dependent on the SVCT-2 protein level. For this purpose, three breast cancer cell lines differentially expressing SVCT-2 were selected. BT20 cells expressing no/low levels of SVCT-2 were transfected with a construct expressing SVCT-2 cDNA or control vector followed by L-ascorbate treatment (Figure 1c). Cells expressing SVCT-2 displayed significantly increased sensitivity to L-ascorbate, but control cells did not (Figure 1c). Next, we examined the effects of SVCT-2 silencing via small interfering RNA (siRNA) on two breast cancer cell lines, Hs578T and T47D, expressing high levels of SVCT-2 (Figures 1d and e). The knockdown effects of the two SVCT-2 siRNAs on Hs578T and T47D were confirmed (Figures 1d and e).

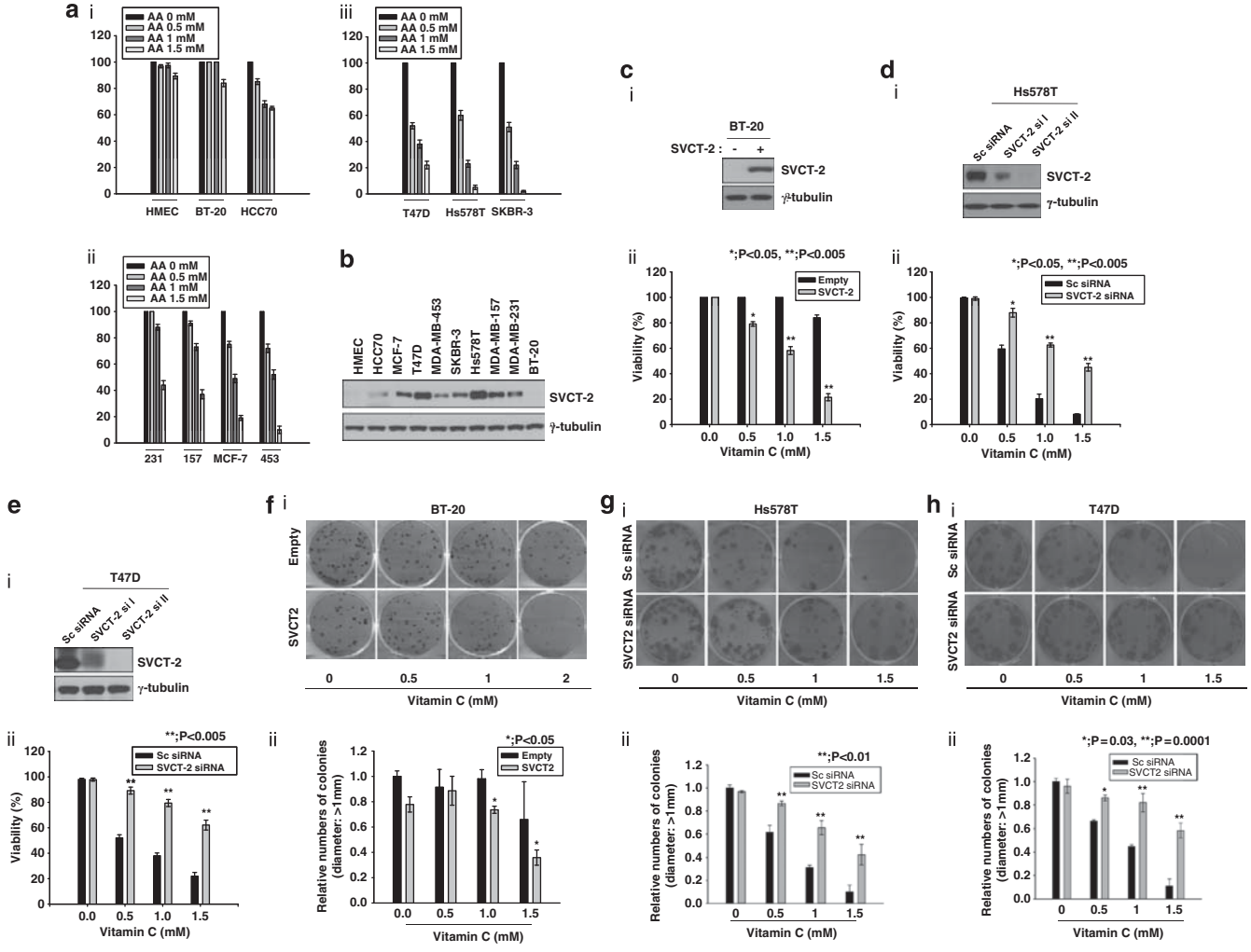

Figure 1. The effects of L-ascorbate (L-L-ascorbic acid, vitamin C) according to SVCT-2 protein level. (**a**) Effect of endogenous SVCT-2 and L-ascorbate on the cell viability of HMECs and breast cancer cell lines. HMECs (i) and breast cancer cell lines (i–iii) were grown in growth medium without vitamin C. The cells were treated with L-ascorbate at the indicated doses for 2 h, washed and incubated for another 18 h in L-ascorbate-free medium. Cell viability was determined by trypan blue exclusion assay. Values are presented as means ± S.E. of three independent experiments performed in duplicate. (**b**) Western blot analysis of endogenous SVCT-2 protein expression in HMEC and breast cancer cell lines. γ-tubulin was used as a loading control. (**c**) BT20, (**d**) Hs578T and (**e**) T47D cells were transfected with SVCT-2-expressing plasmid, control vector (**c**(i)), scrambled siRNA, or SVCT-2-siRNA (**d**, **e**(i)) and analyzed by immunoblotting with SVCT-2 antibody. γ-tubulin was used as a loading control. The cells were treated with L-ascorbate at the indicated doses and cell viability was determined by trypan blue exclusion assay (**c**–**e**(ii)). The error bars represent mean ± s.d. of three separate experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.005$. (**f**–**h**) Colony-forming assay was performed on the cells treated with or without L-ascorbate. The cells were fixed, stained and photographed after 14 days. Shown at (i) are representative images of the colonies formed by each type of cell. The graph shows the number of colonies (ii). The graphs represent mean ± s.d. of three separate experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$.

Cell viability was dramatically increased in SVCT-2 siRNA-treated Hs578T or T47D cells following L-ascorbate treatment (Figures 1d and e), suggesting resistance to L-ascorbate. To further confirm whether differential sensitivity to L-ascorbate is related to SVCT-2 protein expression, we performed colony-forming assay. Colony formation in BT20 cells expressing SVCT-2 was gradually decreased after exposure to L-ascorbate in dose-dependent manner, whereas a decrease in colony formation was almost not observed in BT20 cells that express control vector (Figure 1f). Reversely, colony formation in Hs578T and T47D cells transfected with SVCT-2 siRNA was less decreased than in cells transfected with scrambled siRNA, as a control siRNA, in response to L-ascorbate in dose-dependent manner (Figures 1g and h). In addition, SVCT-2 localization in the membrane after transfection with SVCT-2 siRNA in Hs578T cells or constructs expressing SVCT-2 cDNA in MDA-MB231 cells was additionally confirmed by immunostaining (Supplementary Figure S1). Endogenous SVCT-2 localizing to the membrane in Hs578T cells was not detected after specific siRNA transfection, whereas transfection of SVCT-2 cDNA strongly induced expression in MDA-MB231 cells with low SVCT-2 levels (Supplementary Figure S1). Our results strongly suggest that differences in chemosensitivity to L-ascorbate are dependent on the SVCT-2 protein level.

Differences in ascorbic acid uptake are influenced by the SVCT-2 protein level

As shown in Figure 1, chemosensitivity to L-ascorbate was significantly correlated with the SVCT-2 protein level. Accordingly, we investigated whether differential sensitivity to L-ascorbate arises from variations in L-ascorbate flow into cells, determined by the SVCT-2 protein level. We selected four breast cancer cell lines expressing variable levels of SVCT-2 for analysis. SVCT-2 protein expression in Hs578T and T47D cells was higher than that in HCC70 or MDA-MB231 cells. The four cell lines were treated with 1 mM L-ascorbate in a time-dependent manner (Figure 2a). Notably, the concentrations of L-ascorbate flow into Hs578T and T47D were markedly increased (Figure 2a), but those of HCC70 or MDA-MB231 cells were not (Figure 2a). Next, we examined whether L-ascorbate flow into the cells is dose-dependent. Uptake was gradually increased in all three cell lines treated with various doses of L-ascorbate, including two cancer cell lines and one normal HMEC, but markedly increased in Hs578T cells, compared with that in MDA-MB231 or HMEC cells expressing low levels of SVCT-2 (Figure 2b). These findings imply that the SVCT-2 protein-dependent increased L-ascorbate flow into cells results in decreases of cell viability.

On the basis of the above results, we further analyzed the dependence of L-ascorbate uptake on SVCT-2 using a construct expressing SVCT-2 cDNA or SVCT-2 silencing via siRNA. Initially, MDA-MB231 cells expressing low levels of SVCT-2 were transfected with a construct expressing SVCT-2 cDNA followed by L-ascorbate treatment. Notably, L-ascorbate uptake in SVCT-2-transfected cells was significantly increased (Figure 2c). In contrast, L-ascorbate flow into SCVT-2 siRNA-transfected Hs578T cells that express high SVCT-2 levels was dramatically decreased (Figure 2d). The kinetics of SVCT-2 mediation of L-ascorbate uptake was additionally assayed (Figure 2e). MDA-MB231 cells were dose-dependently transfected with a construct expressing SVCT-2 cDNA (0.5, 1, 2 and 4 μg), before L-ascorbate treatment (Figure 2f). The L-ascorbate level was parallelly increased with increased SVCT-2 cDNA (Figure 2f). Thus, the differential sensitivities to L-ascorbate may result from increased L-ascorbate flow into cells, which is dependent on the SVCT-2 protein level.

Ascorbate influx through SVCT-2 induces an increase in the intracellular reactive oxygen species (ROS) level, and subsequently, cell death

Next, we investigated the effects of L-ascorbate influx on cancer cell viability. Previous reports show that L-ascorbic acid enhances the ROS level.[21,22] We initially analyzed the intracellular ROS level following L-ascorbate treatment in two breast cancer cells differentially expressing SVCT-2 protein. Interestingly, the ROS level in Hs578T cells with high SVCT-2 expression was significantly increased after exposure to L-ascorbate (Figure 3a), but not in MDA-MB231 cells expressing low levels of SVCT-2 (Figure 3c). Next, we examined whether the ROS level is affected by SVCT-2 protein expression. MDA-MB231 cells were transfected with SVCT-2 cDNA in a dose-dependent manner followed by L-ascorbate treatment (Figure 3c). Increased ROS expression was dependent on the SVCT-2 protein level. Conversely, Hs578T cells were transfected with specific siRNA to eliminate endogenous SVCT-2 expression (Figure 3b). The ROS level was increased in scramble siRNA-treated cells but not in SVCT-2-siRNA-treated cells, clearly indicating that L-ascorbate flow into cells through SVCT-2 stimulates an increase in the intracellular ROS level. In view of the above findings, we further examined whether the increased ROS level affects cancer cell viability. The antioxidant, N-acetyl-L-cysteine (NAC), exerted an inhibitory effect on cell death induced by L-ascorbate. Viability of Hs578T cells expressing high levels of SVCT-2 was restored after NAC treatment (Figure 3d). Consistently, viability and colony formation were dramatically increased among L-ascorbate-treated cells in presence of NAC, but not in cells that were treated with L-ascorbate alone (Figure 3d), indicating that NAC affects colony-forming ability of cells that express SVCT-2 through prevention of L-ascorbate-induced ROS production. However, transfection of SVCT-2 cDNA in MDA-MB231 cells expressing low levels of SVCT-2 resulted in increased cell death (Figure 3e), indicating that NAC inhibits cell death through prevention of L-ascorbate-induced ROS production. Consistently, transfection of SVCT-2 cDNA in MDA-MB231 cells expressing low levels of SVCT-2 resulted in decreased colony formation after exposure to L-ascorbate, whereas colony formation in presence of NAC was not decreased in response to L-ascorbate (Figure 3e). Also, catalase, an antioxidative enzyme, was used to further confirm the effect of L-ascorbate-induced ROS production on cell viability and colony formation. Colony formation among catalase stable-expressing cells was significantly increased after exposure to L-ascorbate, but not in cells that express control vector (Figure 3f). Consistently, cell viability was not decreased in cells that express catalase in response to L-ascorbate (Figure 3f). Our results suggest that the L-ascorbate influx dependent on SVCT-2 is a result of increased intracellular levels of ROS, subsequently leading to cell death.

Ascorbate partially induces autophagy, but not caspase-dependent cell death, in human breast cancer cells

The above data show that L-ascorbate triggered cell death in human breast cancer cells. We analyzed the underlying mechanisms involved in cell death process induced by L-ascorbate. Initially, we investigated whether L-ascorbate-induced cell death is caspase-dependent. Caspase-3 was not cleaved in L-ascorbate-treated cells, in contrast to those treated with etoposide, a chemotherapy agent that triggers caspase-dependent cell death (Supplementary Figure S2a). Consistently, the decrease in viability induced by L-ascorbate was not recovered after treatment with Z-VAD, a caspase inhibitor, whereas etoposide-induced cell death was significantly restored (Supplementary Figure S2b), suggesting that L-ascorbate mediates caspase-independent death in human breast cancer cells.

Recent reports show that L-ascorbate induces autophagy in glial cells and non-small cell lung cancer.[23,24] Furthermore, Liang *et al.*[25] demonstrated that Beclin-1, a novel Bcl-2-interacting coiled–coil protein, inhibits tumorigenesis through induction of autophagy. In view of these findings, we investigated the effects of Beclin-1 on caspase-independent cell death by L-ascorbate. The first step was to determine whether L-ascorbate induces

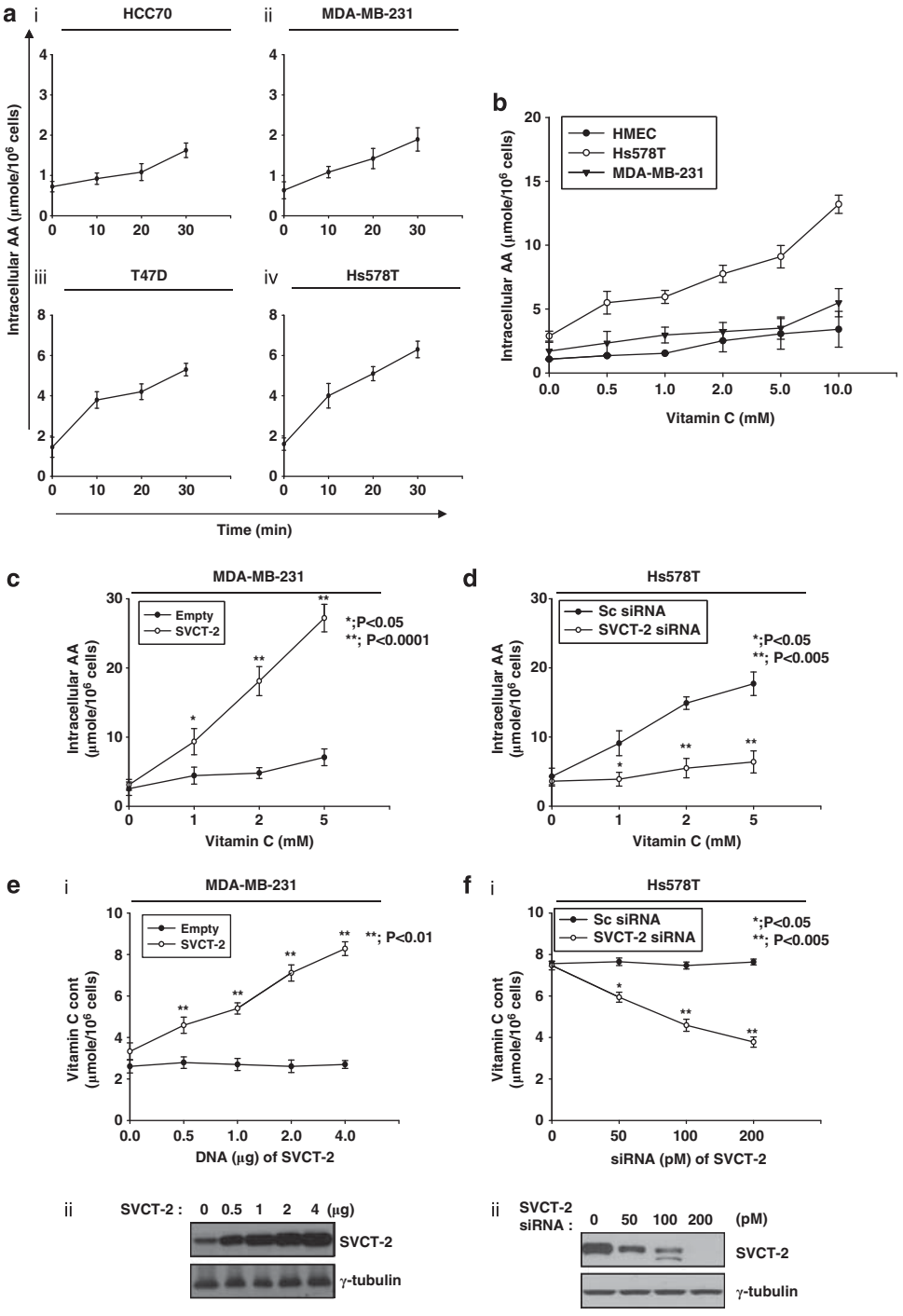

Figure 2. Increased uptake of L-ascorbate is dependent on SVCT-2. (**a**) HCC70, MDA-MB231, T47D and Hs578T cells were treated with 1 mM L-ascorbate for the indicated times. L-ascorbate quantification was analyzed using 2,4-dinitrophenylhydrazine methods. (**b**) HMEC, Hs578T and MDA-MB231 cells were treated with L-ascorbate at the indicated doses for 30 min. (**c**) MDA-MB231 or (**d**) Hs578T cells were transfected with SVCT-2-expressing plasmid, control vector, scrambled siRNA or SVCT-2-siRNA, followed by L-ascorbate at the indicated doses for 30 min, and L-ascorbate concentrations confirmed with the 2,4-dinitrophenylhydrazine assay. Each value represents the mean of three independent experiments ± s.e. (**e**, **f**) Cells transfected with the indicated doses of SVCT-2 cDNA or SVCT-2 siRNA were treated with L-ascorbate. The levels of SVCT-2 were detected by western blot analysis. γ-tubulin was used as a loading control.

Beclin-1 expression (Figure 4). Interestingly, the Beclin-1 level was significantly increased in Hs578T (Figure 4a) and T47D (Figure 4b) cells exposed to L-ascorbate. Next, we analyzed the effects of Beclin-1 siRNA on L-ascorbate-induced cell death. Cell viability was partially recovered in Beclin-1 siRNA-treated cells, but not in scrambled siRNA-treated cells (Figure 4c). Consistently, colony formation was clearly increased in cells that express Beclin-1 siRNA after treatment with L-ascorbate, but not in cells that express scramble siRNA, as a control (Figure 4c). To further ascertain the relationship between L-ascorbate and autophagy, we explored its

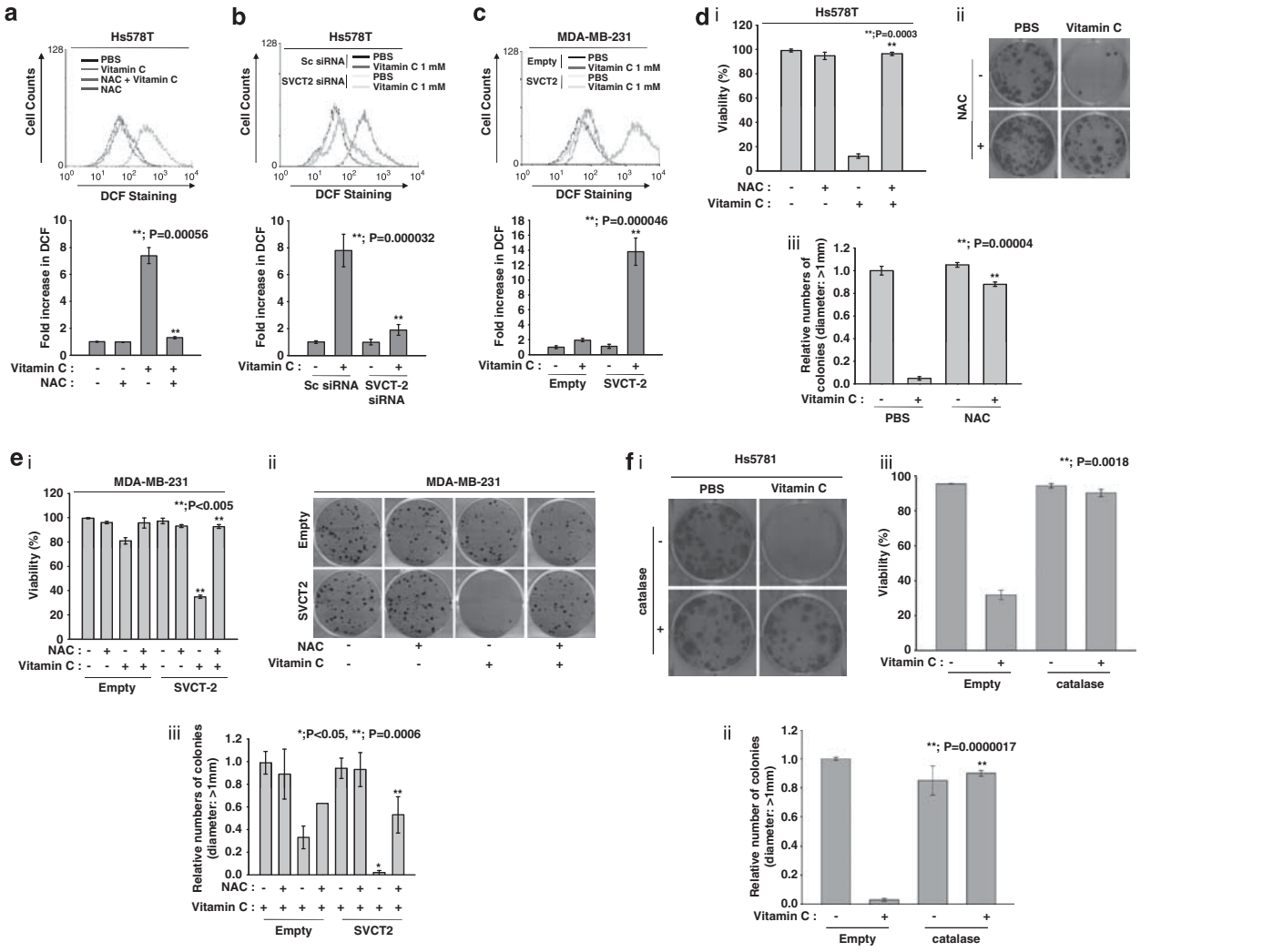

Figure 3. An increase of intracellular ROS level depend on SVCT-2. (**a**) Quantification of ROS levels at a single-cell level in Hs578T by dichlorodihydrofluorescein diacetate (DCF) staining and FACS analysis. Hs578T cells were treated with 1 mM L-ascorbate after pretreatment with 10 mM NAC. The cells washed and incubated with 25 μM of H_2DCF-DA for 30 min, and then were analyzed with FACS. (**b, c**) MDA-MB231 cells were transfected with empty or SVCT-2 DNA for 24 h. Hs578T cells were transfected with scramble siRNA or SVCT-2 siRNA for 48 h. The cells were treated with L-ascorbate, stained with DCF, and then analyzed by FACS. (**d**) Hs578T cells were treated with L-ascorbate after pretreatment with NAC. The cell viability on the cells was determined by trypan blue exclusion assay (i–iii). Colony formation assay on the cells was performed and stained using crystal violet after 14 days. Shown at (ii, iii) are representative images of the colonies formed by each type of cell. The graph shows the number of colonies. (**e**) MDA-MB231 cells were transfected with empty or SVCT-2-expressing plasmid and then treated with vitamin C after pretreatment with or without NAC. Cell viability was determined by the trypan blue exclusion assay. Representative pictures of colony formation on the cells were shown in (ii). The graph shows the number of colonies (iii). (**f**) Hs578T cells were transfected with empty, catalase-expressing plasmid and then treated with L-ascorbate. Cell viability was determined by trypan blue exclusion assay (i). Colony formation assay on the cells was performed and stained using crystal violet after 14 days. Shown at (ii, iii) are representative images of the colonies formed by each type of cell. The graph shows the number of colonies. Data are means ± s.e.m.

effect on LC3 II protein, which has an important role in autophagy.[26] Similar to Beclin-1, the LC3 II protein level was dramatically increased after treatment with L-ascorbate (Figures 4d and e). Consistently, 3-MA, an autophagy inhibitor, partially restored viability and colony formation decreased by L-ascorbate (Figures 4f–h), indicating that L-ascorbate is partially involved in autophagy induction. Interestingly, induction of both Beclin-1 and LC3 II by L-ascorbate was suppressed upon exposure to NAC (Figure 4i), implying that these autophagy-related proteins are upregulated as a result of increased ROS levels. Neither Beclin-1 nor LC3 II was induced by L-ascorbate in cells treated with SVCT-2 siRNA (Figure 4j). These results suggest that L-ascorbate partially induces autophagy through upregulation of Beclin-1 and LC3 II in caspase-independent but SVCT-2-dependent manner.

Ascorbate treatment SVCT-2-dependently decreases tumor growth and SVCT-2 is significantly overexpressed in human breast cancer tissues

On the basis of the above data, we examined the effect of L-ascorbate intraperitoneal administration on the growth of Hs578T and BT20 tumors in nude mice. Tumors in nude mice were treated with either a dose of 1 g L-ascorbate/kg once a day or phosphate-buffered saline. L-ascorbate treatment initiated after tumors reached approximately a size of 20–40 mm^3 in diameter. The growth of Hs578T tumors that highly express SVCT-2 protein was significantly suppressed by L-ascorbate treatment, whereas poor inhibitory effect of L-ascorbate on BT20 tumors, which have low expression of SVCT-2 protein, was observed (Figures 5a and b). An intraperitoneal single administration of L-ascorbate (1 g/kg) in

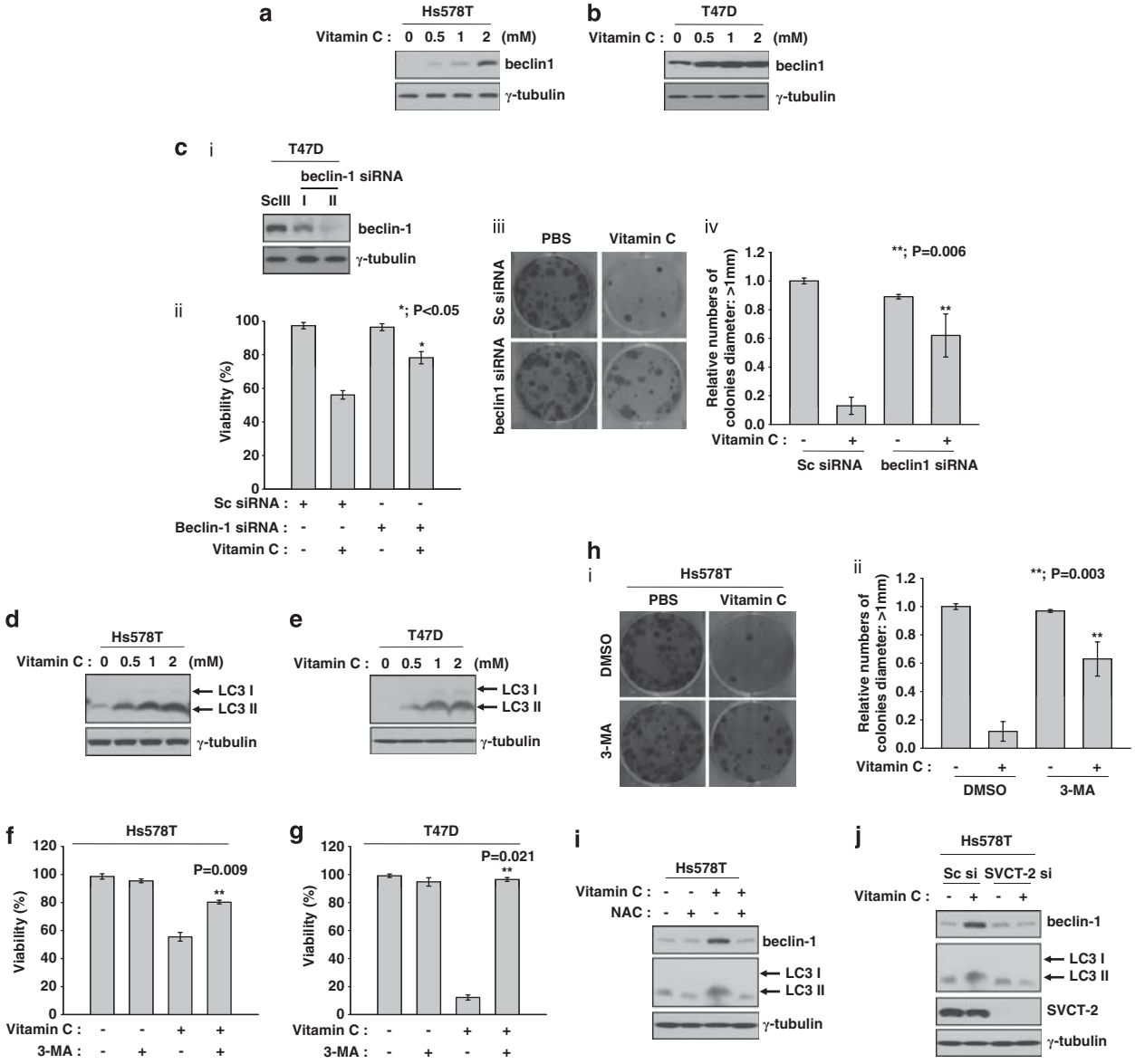

Figure 4. The effect of beclin-1 on cell death induced by L-ascorbate. (**a**) Hs578T or (**b**) T47D cells were treated with L-ascorbate at the indicated doses, and analyzed by immunoblotting with beclin-1 antibody. γ-Tubulin was used as loading controls. (**c**(i)), T47D cells were transfected with Beclin-1 or scrambled siRNA and treated with 0.5 mM L-ascorbate. The beclin-1 protein level was analyzed by immunoblotting using an anti-beclin-1 antibody. γ-Tubulin was used as the loading control. (**c**(ii)) Cell viability was measured using the trypan blue exclusion assay. (**c**(iii, iv)) Colony formation assay on the cells was performed and stained using crystal violet after 14 days. Shown at (iii) are representative images of the colonies formed by each type of cell. The graph shows the number of colonies. Data are means ± s.e.m. (**d**, **e**) Hs578T and T47D cells were treated with L-ascorbate at the indicated doses, and analyzed by immunoblotting with LC3 antibody. γ-Tubulin was used as loading controls. (**f**, **g**) Cells were pretreated with 3-MA, an autophagy inhibitor, for 1 h before treatment with 0.5 mM L-ascorbate. Then, cell viability was analyzed by trypan blue exclusion assy. Data was shown as mean ± s.e.m. from three independent experiments. (**h**(i, ii)) Colony formation assay on the cells was performed and stained using crystal violet after 14 days. Shown at (i) are representative images of the colonies formed by each type of cell. The graph shows the number of colonies. Data are means ± s.e.m. (**i**) Cells pretreated with or without NAC (10 mM) were exposed to 1 mM L-ascorbate. Levels of Beclin-1 and LC3 II were confirmed by western blot analysis. (**j**) Hs578T cells were transfected with SVCT-2-siRNA or scramble siRNA following exposure to 0.5 mM L-ascorbate. Total cell lysates were blotted against the indicated antibodies.

nude mice increased its concentration in the peripheral blood to >2 mM after 30 min. Next, to further confirm whether difference in sensitivity to L-ascorbate on tumor growth is dependent on the level of SVCT-2 protein, SVCT-2-shRNA-expressing Hs578T cells and SVCT-2 stable-expressing BT20 cells were inoculated. Reversely, the growth of SVCT-2-shRNA expressing Hs578T tumors was very slightly decreased after L-ascorbate administration (Figure 5c), whereas inhibitory effect of L-ascorbate on SVCT-2 stable-expressing BT20 tumors was significantly observed (Figure 5d). Thus, xenograft experiments showed that parenteral L-ascorbate could significantly decrease tumor growth dependent on expression levels of SVCT-2 protein.

Furthermore, we confirmed SVCT-2 expression in normal and breast cancer tissues. Surprisingly, the western blotting showed SVCT-2 was highly expressed in 18 of 20 human breast cancers, but only two normal tissues (Figure 5e). We additionally assessed SVCT-2 expression in formalin-fixed and paraffin-embedded tissues obtained from surgical resections of 92 breast cancer

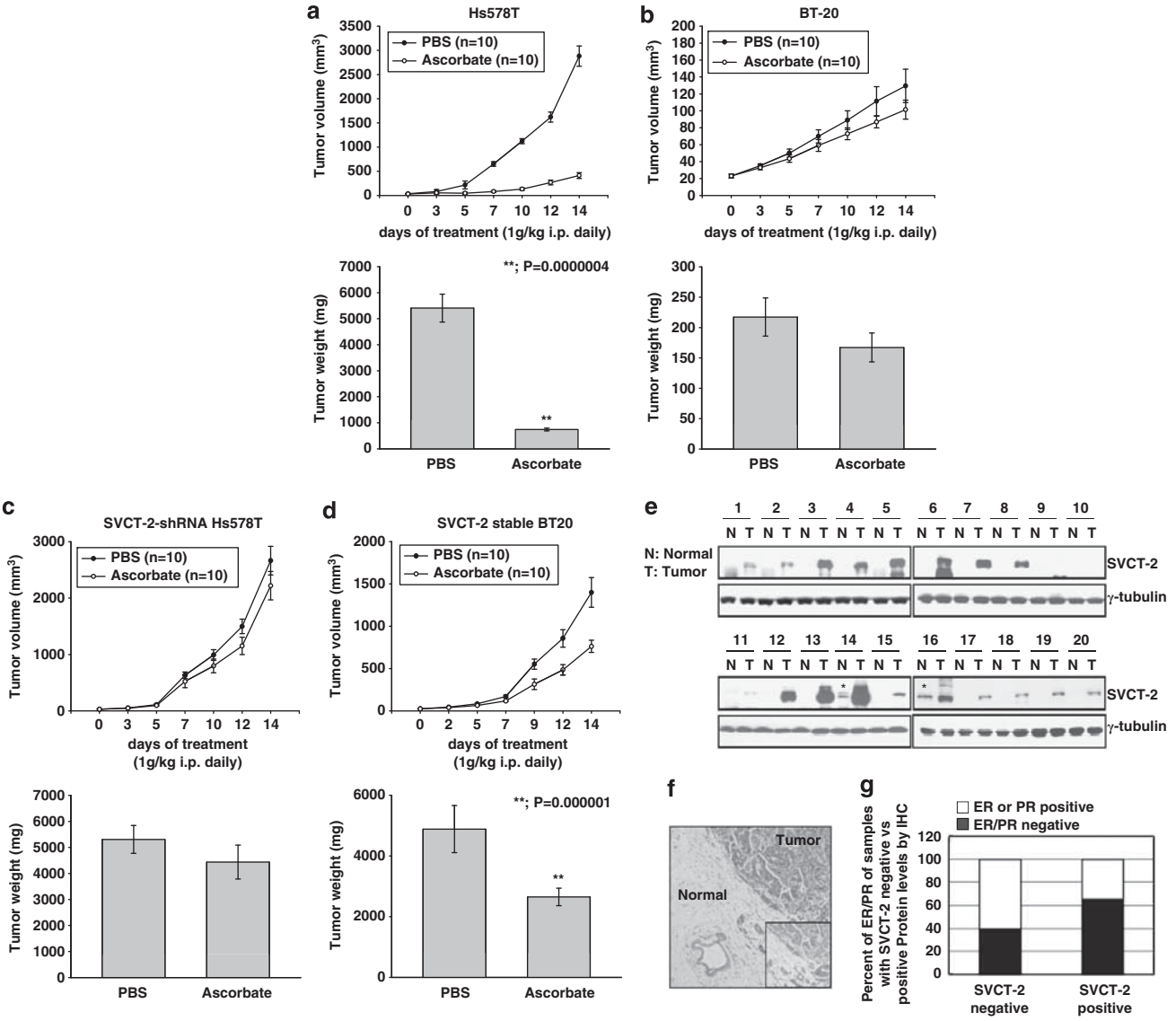

Figure 5. *In vivo* effects of L-ascorbate in Hs578T and BT-20 cells. When (**a**) Hs578T or (**b**) BT20-derived tumors were grown in nude mice to $2 \sim 40\,mm^3$, L-ascorbate was treated to nude mice with dose of 1 g/kg by intraperitoneal injection once a day. The graph shows tumor growth rates. Data are means ± s.e.m. (**c**, **d)** SVCT-2-shRNA expressing Hs578T or SVCT-2 stable-expressing BT20 tumors were prepared and L-ascorbate was also treated as shown in (**a**) and (**b**). *P* values were calculated by the χ^2 test. (**e**) Human breast normal and tumor tissues were obtained from 20 breast cancer patients. Lysates were analyzed by immunoblotting using anti-SVCT-2 antibody. γ-Tubulin was used as the loading control. SVCT-2 was expressed in 1 of 20 normal tissues and 18 of 20 tumor tissues. (*, non-specific). (**f**) Immunoreactivity against SVCT-2 in normal breast cells and tumor cells. SVCT-2 was highly expressed in tumor cells only. (**g**) The statistical correlation between the expression of SVCT-2 and ER/PR in human breast cancer was determined using the χ^2 test (i.e., $P = 0.030$).

patients. Interestingly, significant SVCT-2 expression was detected in 26 (28.3%) of the 92 breast cancer tissue samples. Similar to the data from Figure 1b, SVCT-2 expression was faint or weak in normal breast tissue but strong in breast tumor tissue (Figure 5f). Importantly, ER (estrogen receptor)/PR (progesterone receptor) was significantly associated with SVCT-2 expression in tumor tissue samples. More specifically, >60% of SVCT-2-positive cells showed ER/PR-negative (Figure 5g), to which the conventional chemotherapy is usually applied instead of using herceptin, which is effectively used for ER/PR-positive breast cancer patients. Thus, therapeutic strategies related to SVCT-2 expression could be commanded with more varieties, better effectiveness and less toxicity through particularly attractive adjuvant treatments including L-ascorbate, especially for ER/PR-negative, SVCT-2-positive breast cancer patients.

DISCUSSION

Several studies have focused on the antitumor effects of L-ascorbate to date. However, the mechanism underlying cancer cell susceptibility to L-ascorbate remains to be fully elucidated. Here, we demonstrate for the first time that differences in chemosensitivity to L-ascorbate are dependent on SVCT-2 expression, using one normal HMEC and nine human breast cancer cell lines. Cell line resistance to L-ascorbate was arranged as follows: HMEC > BT20 > MDA-MB231 > HCC70 > MDA-MB157 > MDA-MB453, MCF7 > T47D, Hs578T, SKBR-3 (Figure 1a). Interestingly, this order was in keeping with the SVCT-2 protein level in the individual cell lines (Figure 1b). Conversely, SVCT-1 was highly expressed in cells with low levels of SVCT-2 (Supplementary Figure S3), implying that SVCT-2 is functionally more important than SVCT-1 in cellular

responses triggered by L-ascorbate. Recent studies showing that L-ascorbate flows into cells via SVCT-2, but not SVCT-1, support our findings.[19] In addition, the SVCT-2 protein expression in SKBR-3 is more or less comparable to MCF7, MDA-MB157 as well to MDA-MB231, and SKBR-3 cells undergo almost complete cell death at 1.5 mM L-ascorbate. Therefore, it remains to be elucidated which signaling pathway is responsible for cell death induced by L-ascorbate, excluding SVCT-2.

We also addressed how L-ascorbate flow into cells via SVCT-2 induces death in human breast cancer cells (Figure 4 and Supplementary Figure S2). Recently, M Levine and his colleagues have reported that L-ascorbate acts as a pro-oxidant.[27] Intracellular ROS is produced in changing process of L-ascorbic acid to dehydroascorbic acid (oxidative form of vitamin C).[28] Interestingly, our data also showed that elevated levels of SVCT-2 led to a significant increase in intracellular ROS after exposure to L-ascorbate (Figure 3). In addition, treatment of dehydroascorbic acid, oxidative form of vitamin C, has no effects on cell viability (Supplementary Figure S4).

We further confirmed that L-ascorbate has the inhibitory effect on cancer cells dependent on SVCT-2. L-ascorbate increased intracellular ROS dependent on SVCT-2. The increased levels of ROS by L-ascorbate influx dependent on SVCT-2 were blocked by NAC or catalase, ROS scavenger (Figure 3). Also, an iron chelator, *N*,*N*′-bis (2-hydroxybenzyl) ethlyenediamine-*N*,*N*′-diacetic acid (HBED), prevented ROS induction in response to L-ascorbate (Supplementary Figure S5b). Consistently, treatment of HBED resulted in the prevention from L-ascorbate-induced cell death (Supplementary Figure S5a), suggesting that the L-ascorbate influx dependent on expression levels of SVCT-2 protein results in intracellular ROS production, which induces death of cancer cells.

It was previously reported that Bcl-xL was revealed to inhibit mitochondrial ROS production.[29] Here, we also confirmed that colony formation and viability partially increased in cells expressing Bcl-xL exposed to L-ascorbate, but not in cells expressing control vector (Supplementary Figure S6a and b). Consistently, viability in cells exposed to Mito Q or Mito-tempol, as a mitochondria-targeted antioxidant, more increased than in cells treated with L-ascorbate alone (Supplementary Figure S7a), implying that antioxidants function of Bcl-2 may be important for inhibiting mitochondrial ROS production. Surprisingly, these results are correlated with the alteration of redox state in both Orp1- and Grx1-based probes (Supplementary Figure S7c). In addition, transfection of glutathione peroxidase plasmids also resulted in the protection from L-ascorbate-induced cell death (Supplementary Figure S7b). Thus, L-ascorbate induces cell death through alteration of redox state of cytosol and mitochondria.

Importantly, ROS triggered autophagy through induction of Beclin-1, which inhibits tumorigenesis, and of LC3 II (Figure 4). Thus, L-ascorbate flowed into cells through SVCT-2 results in the increased ROS levels and subsequently leads to induction of both beclin-1 and LC3 II (Figure 4j). Also, we further confirmed the relationship between L-ascorbate and autophagy using three recommended methods of guidelines.[30] Knockdown of ATG5 or ATG7 resulted in prevention from L-ascorbate-induced cell death (Supplementary Figure S8a and b). Moreover, treatment of L-ascorbate resulted in the induction of autophagic vacuoles and the decrease of p62 level, indicating activation of autophagic flux (Supplementary Figure S8c and d). Thus, autophagy may be one of the cell death mechanisms induced by L-ascorbate treatment.

In addition, we showed the effect of L-ascorbate in the presence and absence of functional SVCT-2 in mouse embryonic cell line, NIH3T3 (Supplementary Figure S9). Similar to human breast cancer cells, expression levels of SVCT-2 protein also sensitize NIH3T3 to L-ascorbate damage by inducing beclin-1 and LC3 II.

In view of these results, we evaluated the effect of L-ascorbate on tumor growth in nude mice. L-ascorbate treatment significantly decreased growth of Hs578T tumor that highly expresses SVCT-2 protein, whereas it had poor effects on growth of BT20 tumor that lowly express SVCT-2 protein (Figures 5a and b). Furthermore, the growth of Hs578T tumors that was suppressed by L-ascorbate treatment was revered after exposure to NAC (Supplementary Figure S10a), whereas the growth of BT20 tumors that express no/low levels of SVCT-2 was not affected by NAC or/and L-ascorbate administration (Supplementary Figure S10b), suggesting that L-ascorbate could decrease tumor growth through increase of intracellular ROS level dependent on expression levels of SVCT-2 protein. These results were paralleled with effects of L-ascorbate already shown on various human breast cancer cells.

More importantly, we evaluated SVCT-2 expression in normal and breast cancer tissues. Surprisingly, SVCT-2 was highly expressed in human breast cancers, but only weakly in normal tissues (Figure 5). In addition, SVCT-2 was expressed during the late stages of breast cancer, but not during early stages, indicating its involvement in the latter stages of breast cancer development (Supplementary Table S1). However, we are uncertain whether SVCT-2 contributes to breast cancer development and tumorigenesis. Importantly, a significant association between ER/PR and SVCT-2 expression was observed in tissue samples from breast cancer patients (Figure 5g). ER/PR-negative tumor tissues in SVCT-2-positive samples are 17 (65.4%) of 26. This finding suggests the potential of vitamin C therapy for breast cancer patients that are ER/PR-negative. However, SVCT-2 expression was unrelated to lymph node metastasis or tumor size (Supplementary Table S1).

Thus, differences in chemosensitivity to L-ascorbate treatment are dependent on SVCT-2 levels in multiple human breast cancer cells. These findings suggest that this new treatment modality that relies on the SVCT-2 protein level may be an attractive adjuvant to conventional chemotherapeutic options for breast cancer treatment.

MATERIALS AND METHODS

Cell culture, agents, plasmid DNA, transfection, stable cell line

Multiple human breast cancer cell lines were cultured in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY, USA) containing high glucose and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml). HMEC were cultured with HMEC basal medium (Invitrogen, Carlsbad, CA, USA) containing HMEC supplements. L-ascorbic acid, DTT, 3-MA, etoposide, Z-VAD, puromycin and NAC were purchased from Sigma (St Louis, MO, USA). Human cDNA encoding SVCT-2 or myc-tagged catalase was acquired from Origene (Rockville, MD, USA). We additionally constructed myc-tagged SVCT-2 from SVCT-2-expressing plasmid using myc-tagged pcDNA 3.1 vector. Lipofect-AMINE 2000 (Invitrogen) was employed for transient transfection into BT20 and MDA-MB231 cells. SVCT-2 stable-expressing BT20 cells were prepared by transfecting BT20 cells with SVCT-2 plasmid using Lipofect-AMINE 2000 and then cells were selected with blasticidin (invitrogen) 10 μg/ml at 4 weeks. Hs578T-SVCT-2 shRNA stable cells were transfected using origene SVCT-2 shRNA kit and then cells were selected with puromycin at 2 weeks. Catalase-expressing Hs578T cells were prepared using pBabe-puro from pCMV-entry-myc-catalase.

RNA Interference

Hs578T and T47D human breast cancer cells were transiently transfected with scrambled or SVCT-2-(150 pmol/60 mm dish, SVCT-2 siRNA I: 5′-GGAAGAAGGGUGUGGGCAA-3′; siRNA II: 5′-GGAAAGAGGAAUCCGGAAA-3′) or beclin-1 siRNA (150 pmole/60 mm dish, beclin-1 siRNAI: 5′-UUCAACACUCUUCAGCUCAUCAUCC-3′; beclin-1 siRNA II: 5′-CAGUUUGGCACAAUCAAUA-3′) using Lipofect-AMINE 2000 (Invitrogen). Scrambled siRNA (SVCT-2: 5′-GGUAGAACGGAGAGGGGUA-3′; beclin-1: 5′-GCUUUGGGAUAUCAUAGCGAUGAAU-3′) was obtained from Proligo LLC (Boulder, Co, USA).

Cell viability analysis

Control cells or those expressing SVCT-2, the SVCT-2-siRNA (knockdown), or scrambled siRNA (control) were seeded at 10^5 cells per 60 mm dish for 24 h before treatment with L-L-ascorbic acid, cells were subsequently

treated with L-L-ascorbic acid for 2 h and washed and incubated with L-ascorbic acid-free medium for another 18 h. Cell viability was determined by trypan blue exclusion using at least 300–500 cells for each group.

Western blot analysis

Total cell protein (20 μg) was subjected to SDS–polyacrylamide gel electrophoresis and transferred to PolyScreen membrane (NEN, Boston, MA, USA). Membranes were blocked with 5% non-fat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween 20) buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) and probed with antibodies. The following antibodies were employed: anti-SVCT-2 (kindly provided from Norio Itoh; Osaka University), anti-SVCT-1 (Alpha Diagnostic International Incorporation), anti-beclin-1, anti-γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LC3, anti-p62 and anticleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA). Primary antibodies were detected with goat anti-mouse, goat anti-rabbit or donkey anti-goat horseradish peroxidase-conjugated secondary antibody, and proteins were visualized with the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

L-ascorbate quantification

Total L-ascorbate quantification was performed using the 2,4-dinitrophenylhydrazine method, as described previously.[31]

Measurement of ROS levels

Cells were incubated with 25 μM H_2DCF-DA (Molecular Probe, Eugene, OR, USA) for 30 min, washed with phosphate-buffered saline, trypsinized, and collected in 1 ml of phosphate-buffered saline. Fluorescence-stained cells were transferred to polystyrene tubes with cell strainer caps (Falcon) and subjected to fluorescence-activated cell sorting (FACS; Beckton Dickson FACScan, San Jose, CA, USA) using Cell Quest 3.2 (Beckton Dickson) software for analysis.[32]

Clonogenic survival assay

Cells (2×10^2) per well were seeded in a 6-well plate. Cells were treated with L-ascorbate at the indicated doses for 18 h after pretreatement with NAC or 3-MA and then cultured for 14 days. Colonies were fixed with 10% formalin and then stained with 0.01% crystal violet solution. Colony numbers were counted by diameter 1 mm.

Tumor xenograft

We used a 5-week-old female Balb-C nude mice from Japan SLC laboratory. All experiments were approved by the Animal Experimentation Committee at Seoul National University. Mice were subcutaneously injected with 5×10^6 Hs578T or Hs578T-shRNA stable or BT20 or BT20 SVCT-2 stable cells resuspended in matrigel on the right flank. When tumor volume had reached 30 mm^3, vitamin C (1 g/kg) or phosphate-buffered saline was injected intraperitoneally. Tumor size (length $\times$ width$^2 \times 0.5$) was measured one time per 3 days after treatment with L-ascorbate. At 2 weeks, tumors were excised and weighed.

Statistical analysis

Data were statistically analyzed using a two-tailed Student's *t*-test, and the level of significance stated in the text was based on *P* values and we considered a *P* value <0.05 as significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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