Original Contribution

L-Ascorbic acid can abrogate SVCT-2-dependent cetuximab resistance mediated by mutant KRAS in human colon cancer cells


Innovative Cancer Research, Asan Institute for Life Science, Republic of Korea
Department of Oncology, University of Ulsan College of Medicine, Asan Medical Center, 88 Olympicro-43gil, Songpa-gu, Seoul, Republic of Korea
Department of Convergence Medicine, University of Ulsan College of Medicine, Asan Medical Center, 88 Olympicro-43gil, Songpa-gu, Seoul, Republic of Korea
Department of Anatomy and Tumor Immunity Medical Research Center, Seoul National University College of Medicine, Seoul 110-744, Republic of Korea

Abstract

Colon cancer patients with mutant KRAS are resistant to cetuximab, an antibody directed against the epidermal growth factor receptor, which is an effective clinical therapy for patients with wild-type KRAS. Numerous combinatorial therapies have been tested to overcome the resistance to cetuximab. However, no combinations have been found that can be used as effective therapeutic strategies. In this study, we demonstrate that L-ascorbic acid partners with cetuximab to induce killing effects, which are influenced by sodium-dependent vitamin C transporter 2 (SVCT-2) in human colon cancer cells with a mutant KRAS.

L-Ascorbic acid treatment of human colon cancer cells that express a mutant KRAS differentially and synergistically induced cell death with cetuximab in a SVCT-2-dependent manner. The ectopic expression of SVCT-2 induced sensitivity to L-ascorbic acid treatment in human colon cancer cells that do not express SVCT-2, whereas the knockdown of endogenous SVCT-2 induced resistance to L-ascorbic acid treatment in SVCT-2-positive cells. Moreover, tumor regression via the administration of L-ascorbic acid and cetuximab in mice bearing tumor cell xenografts corresponded to SVCT-2 protein levels. Interestingly, cell death induced by the combination of L-ascorbic acid and cetuximab in mice bearing tumor cell xenografts corresponded to SVCT-2 protein levels. Interestingly, cell death induced by the combination of L-ascorbic acid and cetuximab resulted in both apoptotic and necrotic cell death. These cell death mechanisms were related to a disruption of the ERK pathway and were represented by the impaired activation of RAFs and the activation of the ASK-1–p38 pathway. Taken together, these results suggest that resistance to cetuximab in human colon cancer patients with a mutant KRAS can be bypassed by L-ascorbic acid in an SVCT-2-dependent manner. Furthermore, SVCT-2 in mutant KRAS colon cancer may act as a potent marker for potentiating L-ascorbic acid co-treatment with cetuximab.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world. It causes approximately 609,000 human deaths every year, which accounts for 8% of all cancer deaths. The overall five-year survival rate for CRC patients is 65%, although this differs depending on the stage at diagnosis [1]. Typical biological therapies for CRC are combination chemotherapies. Cetuximab, an epidermal growth factor receptor (EGFR) monoclonal antibody, is known to be an effective clinical therapy for CRC patients with wild-type but not KRAS-mutant tumors [2,3]. However, the clinical use of cetuximab is limited due to high rates of resistance.
addition, only 10–20% of cancer patients show a positive response to anti-EGFR treatment [4]. To overcome cetuximab resistance in KRAS-mutant tumors, combination chemotherapies incorporating cetuximab with panitumabum or celecoxib have been studied [5], and the ERBB2 or PI3K pathways are speculated to be targets for the combination therapy. The mechanism for overcoming resistance to cetuximab still remains to be elucidated; therefore, more effective strategies need to be developed.

One effective anti-cancer agent is L-ascorbic acid, which is commonly known as vitamin C. The administration of daily doses of 10 g showed a beneficial effect on cancer chemotherapy, and the induction of cancer cell apoptosis and cell cycle arrest by L-ascorbic acid was identified in brain, breast, prostate, and stomach cancer [6–9]. Additionally, it is an essential nutrient and cofactor for human metabolism. It plays an important role in protecting cells against oxidative stress, including cancer, which is influenced by the sodium-dependent vitamin C transporter (SVCT) family [10]. The vitamin C transporter SVCT has two subtypes, SVCT-1 and SVCT-2. The expression of SVCT-1 is detected in the intestinal epithelium and the liver, and SVCT-2 has also been identified in various organs. Notably, vitamin C protects cells against oxidative stress via the uptake of metabolically active SVCT-2 [11, 12]. SVCT-2 was recently demonstrated to be a key protein for vitamin C uptake in the liver, and genetic variants are associated with head and neck cancer [13]. Our previous study demonstrated that L-ascorbic acid treatment is associated with the chemosensitivity of SVCT-2 in breast cancer [14]. According to recent reports, DHA and an oxidized form of vitamin C were effective therapeutic agents for KRAS- and BRAF-mutant colorectal cancer cells [15]. These findings support the notion that SVCT-2 is an important functional protein associated with cancer.

The goal of this investigation was to eliminate resistance against cetuximab in CRC patients with a mutant KRAS. We first examined whether L-ascorbic acid activity was dependent on SVCT-2 expression in human colon cancer cells with a mutant KRAS. We focused on the induction of cell death and the anti-cancer effects in vitro and in vivo upon co-treatment with cetuximab and L-ascorbic acid. This combined treatment is thought to primarily block the MAPK pathway followed by apoptosis and necrosis. Our data demonstrated additional therapeutic effects of L-ascorbic acid against cetuximab resistance, and our observations suggest that SVCT-2 is a potent marker for the treatment with L-ascorbic acid and cetuximab in KRAS-mutant CRC patients.

2. Materials and methods

2.1. Cell culture and reagents

The DLD1, SW480, SW620, HCT8 and HCT116 colorectal cancer cell lines were purchased from KCLB (Korean Cell Line Bank, Seoul, KOREA). All cell lines were maintained with RPMI-1640 medium (WELGENE) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA) and 100 µg/ml penicillin/streptomycin in a 5% CO2 incubator at 37 °C. L-Ascorbic acid (Sigma, St. Louis, MO, USA), cetuximab (Erbitux; Merck, Germany) and necrostatin-1 (Selleckchem, Houston, TX) were diluted in PBS (L-ascorbic acid) and DMSO before the experiment.

2.2. Cell death and flow cytometry analysis

The cells were treated with L-ascorbic acid at the indicated doses for 6 h, and cell death was determined using the trypan blue exclusion method. The cells were seeded in 60-mm dishes at a density of 4 × 10^3 cells for a flow cytometry assay. Then, the cells were treated with L-ascorbic acid and cetuximab. The cells were washed with PBS, trypsinized and stained with propidium iodide and annexin-V staining solutions (BD Biosciences, San Jose, CA, USA) and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.3. Plasmids, siRNA and transfection

Human cDNA encoding SVCT-2 or myc-tagged catalase was acquired from Origene (Rockville, MD, USA). We additionally constructed myc-tagged SVCT-2 from a SVCT-2-expressing plasmid using a myc-tagged pcDNA 3.1 vector. The following sequences were used for the siRNA transfection: Scramble, 5′-GCCCAUUC-AAGCUUACGGAUUG and SVCT2, 5′-GGAAGAGCGGAAUCCGAAAUU. All DNA and siRNA transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

2.4. Immunoblotting analysis

Total cellular protein, at 20 µg per well, was resolved by 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for western blot analysis and then transferred to Immobilon-P membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS-T; 20 mM Tris–HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20) and then incubated with primary antibodies (anti-SVCT-2, anti-CRAF, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-myc, anti-cleaved caspase-3, anti-AKT, anti-phospho AKT, anti-ERK1/2, anti-phospho ERK1/2, anti-BRAF, anti-phospho BRAF, anti-phospho CRAF, anti-MEK, anti-phospho MEK, anti-ASK-1, anti-phospho ASK-1, anti-p38, anti-phospho p38 (Cell Signaling, Beverly, CA, USA), and anti-HMGB1 (Abcam, Cambridge, MA, USA) at 4 °C for 12 h. The primary antibodies were detected using goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies and enhanced via chemiluminescence detection (Amersham, Buckinghamshire, UK).

2.5. Colony formation assay

The cells were harvested with trypsin-EDTA and counted using a hemocytometer. Next, the cells were diluted and seeded at 150 cells per well in six-well plates. After a 12 h incubation, the cells were treated for 6 h with L-ascorbic acid and cetuximab and then continuously incubated in fresh medium in a 5% CO2 incubator at 37 °C. After a 14-day incubation, the cells were washed with PBS, fixed with 10% formaldehyde, and stained with 0.05% crystal violet for 20 min at room temperature. The visible colonies were counted.

2.6. ROS level measurements

The cells were incubated with 20 µM H2DCF-DA (Molecular Probe, Eugene, OR, USA) for 30 min, washed with PBS, trypsinized, and collected in 1 ml of PBS. Fluorescence-stained cells were transferred to polystyrene tubes with a cell strainer (Falcon) and subjected to fluorescence-activated cell sorting (FACS; Becton Dickinson FACScan, San Jose, CA, USA) using FlowJo V.10.1 (FlowJo, LLC) software for the analysis.

2.7. Mouse xenograft model

Athymic nude mice (5-week-old females) were obtained from the Japan SLC Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee at the ASAN Laboratory of Animal Research. The mice were subcutaneously injected with 5 × 10^6 SW620 or HCT116 cells in the right flank. Once the tumors reached 50mm^3, the mice were administered their
respective treatments (vehicle, cetuximab, vitamin C and the combination of cetuximab and vitamin C). The cetuximab dose for all experiments was 50 mpk intraperitoneally twice weekly. The dose of vitamin C for all experiments was 0.5 g/kg intraperitoneally daily for two weeks. Tumor size \( \left( \text{length} \times \text{width}^2 \times 0.5 \right) \) was measured one time every 3 days. At two weeks, the tumors were excised, weighed, and analyzed by immunohistochemistry.

2.8. Statistical analysis

All data were statistically analyzed with a two-tailed Student’s t-test. P-values < 0.05 were deemed significant.

3. Results

3.1. L-Ascorbic acid selectively induces cell death in mutant KRAS human colon cancer cells that are resistant to cetuximab in an SVCT-2-dependent manner

To investigate the effects of L-ascorbic acid, we first examined the protein levels of SVCT-2 in various human colon cancer cells that express mutant KRAS. The HCT8 and HCT116 cells did not express the SVCT-2 protein, whereas the DLD1, SW480, and SW620 cells showed a high expression of the SVCT-2 protein (Fig. 1A). Based on the above data, we examined the inhibitory effects of L-ascorbic acid and/or cetuximab on these cells in the context of SVCT-2. Treatment of these cells with L-ascorbic acid and/or cetuximab resulted in increased cell death in the SVCT-2-expressing cell lines DLD1, SW480, and SW620 but not in the HCT8

![Fig. 1. L-ascorbic acid (vitamin C, VitC) sensitizes SVCT-2-positive/KRAS-mutant human colon cancer cell lines to cetuximab (CTX). (A) Among the 5 KRAS-mutant colon cancer cell lines, HCT8 and HCT116 do not express SVCT-2, whereas DLD1, SW480 and SW620 show high levels of SVCT-2 expression. (B) The graph shows whether cell death was induced with 0.5 μM cetuximab with or without L-ascorbic acid. Compared with the SVCT-2-negative cell lines (HCT8, HCT116), the SVCT-2-positive cell lines (DLD1, SW480 and SW620) showed significantly increased cellular death under the combinatorial treatment with cetuximab and L-ascorbic acid. (C) A colony-forming assay with 0.5 μM cetuximab and 0.7 mM L-ascorbic acid treated cells. The numbers of colonies were significantly decreased in the L-ascorbic acid and cetuximab-treated SVCT-2-positive cell lines. However, the numbers of colonies did not affect the L-ascorbic acid or cetuximab-treated SVCT-2-negative cells (data not shown).]
and HCT116 cell lines, which did not express SVCT-2 (Fig. 1B). To further investigate whether the combinational effects of L-ascorbic acid and cetuximab are related to SVCT-2 protein expression, we performed a colony-forming assay. Colony formation in three colon cancer cell lines, DLD1, SW480, and SW620, expressing the endogenous SVCT-2 was dramatically decreased after co-treatment with L-ascorbic acid and cetuximab (Fig. 1C). In addition, there were no responses to cetuximab alone in these cells, independent of SVCT-2, implying that the combinatorial effects of L-ascorbic acid and cetuximab may be related to SVCT-2 protein expression.

To ascertain whether L-ascorbic acid is related to SVCT-2 protein expression in mutant KRAS colon cancer cells that are resistant to cetuximab, we initially examined the effects of L-ascorbic acid on these cells and whether they were dependent on SVCT-2. To this end, we transfected HCT8 and HCT116 cells, which do not express SVCT-2, with a construct containing SVCT-2 cDNA or a control vector followed by L-ascorbic acid treatment. Both cell lines expressing ectopic SVCT-2 cDNA exhibited significantly increased sensitivity to L-ascorbic acid, but the control cells did not (Fig. 2A). Consistently, the cleavage of caspase-3 was also induced in a manner that was dependent on SVCT-2 protein expression.

Next, we examined the effects of SVCT-2 silencing via small interfering RNA (siRNA) on the three mutant KRAS colon cancer cell lines, DLD1, SW480, and SW620, which are resistant to cetuximab. Cell death rate decreased in the SVCT-2 siRNA-treated colon cancer cell lines, consistent with the findings in Fig. 1B.

**Fig. 2.** L-Ascorbic acid induces cell death in a manner dependent on SVCT-2 expression. (A) The SVCT-2-negative cell lines were transfected with SVCT-2 (myc-tagged) and then treated with L-ascorbic acid. After the transfection of SVCT-2, cell death was remarkably increased under L-ascorbic acid treatment. (B) The cell lines that showed high levels of SVCT-2 expression, including DLD1, DW480 and SW620, were transfected with the SVCT-2 siRNA and treated with L-ascorbic acid. By silencing SVCT-2, cell death was significantly decreased, and the cleavage of caspase-3 expression was reduced.
cells following L-ascorbic acid treatment (Fig. 2B). In addition, cleaved caspase-3 was not induced in any of the tested cells. These results strongly suggest that differences in the sensitivity to L-ascorbic acid are dependent on the SVCT-2 protein levels in mutant KRAS human colon cancer cells that are resistant to cetuximab.

3.2. Co-treatment with L-ascorbic acid and cetuximab induces necrotic cell death as well as apoptosis

As shown in Fig. 1, treatment with L-ascorbic acid and cetuximab induced cell death in mutant-KRAS human colon cancer cells that expresses SVCT-2. (A) FACS analysis after an 8 h treatment with L-ascorbic acid and 0.5 μM cetuximab in a SVCT-2-positive CRC cell line. The combined treatment of L-ascorbic acid and cetuximab augments membrane (Annexin V) and nuclear (PI) damage. In particular, in the combinatorial treatment group, PI single-stained areas (necrotic areas) were remarkably increased. (B) Caspase-3 cleavage after treatment for 8 h with both 0.5 μM cetuximab and 0.7 mM L-ascorbic acid. The activation (cleavage) of caspase-3 is observed only in SVCT-2-positive cell lines. (C) Changes in cellular death after treatment with the caspase inhibitor Z-VAD. The cells were pre-incubated with 100 μM Z-VAD for 20 min and treated with the combination therapy. Cell death was slightly reduced under Z-VAD treatment. (D) Changes in necrosis following drug treatment. Under Z-VAD treatment, HMGB1 (necrosis marker) cleavage remains unchanged in the SVCT-2-positive CRC cell lines. (E) Changes in cell death after co-pre-treatment with 30 μM Nec-1 (necrostatin-1, necrosis inhibitor) and 100 μM Z-VAD. By treating with both Nec-1 and Z-VAD, the SVCT-2-positive cell lines fully escaped from the cetuximab and L-ascorbic acid-induced cell death.

Fig. 3. Co-treatment with L-ascorbic acid and cetuximab induces both necrotic and apoptotic cell death in a mutant KRAS colon cancer cell line that expresses SVCT-2. (A) FACS analysis after an 8 h treatment with L-ascorbic acid and 0.5 μM cetuximab in a SVCT-2-positive CRC cell line. The combined treatment of L-ascorbic acid and cetuximab augments membrane (Annexin V) and nuclear (PI) damage. In particular, in the combinatorial treatment group, PI single-stained areas (necrotic areas) were remarkably increased. (B) Caspase-3 cleavage after treatment for 8 h with both 0.5 μM cetuximab and 0.7 mM L-ascorbic acid. The activation (cleavage) of caspase-3 is observed only in SVCT-2-positive cell lines. (C) Changes in cellular death after treatment with the caspase inhibitor Z-VAD. The cells were pre-incubated with 100 μM Z-VAD for 20 min and treated with the combination therapy. Cell death was slightly reduced under Z-VAD treatment. (D) Changes in necrosis following drug treatment. Under Z-VAD treatment, HMGB1 (necrosis marker) cleavage remains unchanged in the SVCT-2-positive CRC cell lines. (E) Changes in cell death after co-pre-treatment with 30 μM Nec-1 (necrostatin-1, necrosis inhibitor) and 100 μM Z-VAD. By treating with both Nec-1 and Z-VAD, the SVCT-2-positive cell lines fully escaped from the cetuximab and L-ascorbic acid-induced cell death.
cell death in mutant KRAS colon cancer cells that express the SVCT-2 protein. Based on the above data, we next investigated whether the combination-induced cell death is caspase-3-dependent. Co-treatment with L-ascorbic acid and cetuximab led to the cleavage of caspase-3 in the mutant KRAS colon cancer cells expressing the SVCT-2 protein but not in cells that did not express SVCT-2 (Fig. 3B), which indicates that the combinatorial treatment induces caspase-dependent apoptotic cell death. To further confirm this result, we investigated whether Z-VAD, a caspase inhibitor, can inhibit caspase-dependent apoptotic cell death. Interestingly, Z-VAD treatment partially inhibited cell death induced by the combinatorial treatment. However, the expression of HMGB1, a signal of necrosis, was induced by the combination treatment and was not suppressed by Z-VAD treatment (Fig. 3C), implying that the combination treatment may induce necrosis as another mechanism of cell death.

To investigate whether the combination of L-ascorbic acid and cetuximab induces necrotic cell death in the mutant KRAS colon cancer cells expressing the SVCT-2 protein, the cells were treated with necrostatin-1 (nec-1), an inhibitor of necroptosis, followed by the combinatorial treatment. Necrostatin-1 partially inhibited the cell death induced by the combinatorial treatment, similar to the Z-VAD treatment (Fig. 3E). In particular, co-treatment with necrostatin-1 and Z-VAD almost completely blocked the combination-induced cell death. Consistently, the expression of HMGB1, a signal of necrosis, was suppressed in response to necrostatin-1 after exposure to the combinatorial treatment. These results suggest that the combination of L-ascorbic acid and cetuximab induces necrotic and apoptotic cell death in the mutant KRAS colon cancer cells expressing the SVCT-2 protein.

**Fig. 4.** Co-treatment with L-ascorbic acid and cetuximab induces cell death through RAFs and the MAPK pathway. (A) The combinatorial treatment of 0.7 mM L-ascorbic acid and 0.5 μM cetuximab suppresses the activation of the MAPK pathway in a SVCT-2-dependent manner. These cells were harvested and analyzed by a western blot to assess phospho-MEK and ERK. The phosphorylation of MEK and ERK is remarkably decreased in the SVCT-2-positive cell lines but not in the SVCT-2-negative cell lines (HCT8, HCT116). (B) The activation of RAFs was blocked under the co-treatment. In the SVCT-2-positive cell lines, the phosphorylation of the RAFs (particularly C-RAF) was blocked after treatment with cetuximab and L-ascorbic acid.
3.3. Co-treatment with L-ascorbic acid and cetuximab leads to the inhibition of phospho-BRAF, CRAF, and the MAPK pathway

Based on a report demonstrating that the resistance mechanisms to cetuximab are related to activation of the RAF protein and the subsequent activation of the MAPK pathway [16], we examined the inhibitory effects of L-ascorbic acid and cetuximab on the activation of pathway-related molecules. Treatment with L-ascorbic acid and cetuximab significantly blocked the phosphorylation of ERK and MEK in the endogenous SVCT-2-expressing colon cancer cell lines DLD1, SW480, and SW620, whereas the phosphorylation of these proteins was not inhibited in the HCT8 and HCT116 cells that do not express SVCT-2 following treatment with both drugs (Fig. 4A). Furthermore, the phosphorylation of BRAF and CRAF was dramatically inhibited in parallel with that of ERK and MEK after exposure to L-ascorbic acid and cetuximab (Fig. 4B), suggesting that L-ascorbic acid and cetuximab induce cell death through the inhibition of RAF and the MAPK pathway in the mutant KRAS colon cancer cells that are resistant to cetuximab and dependent on SVCT-2. Additionally, we investigated the functional mechanism of the combinational effects. These results demonstrate that cetuximab and L-ascorbic acid induce ROS production...
and inhibit CRAF activation. Moreover, ASK-1 activation induces the phosphorylation of p38, which then contributes to cellular apoptosis in the SVCT-2 expressing cell line (Supplementary Figs. 2 and 3) [17]. However, the SVCT-2-negative cells did not induce the ASK-1–p38 pathway.

3.4. The combination of L-ascorbic acid and cetuximab decreases tumor growth in a SVCT-2-dependent manner

Based on the above results, we examined the inhibitory effects of L-ascorbic acid i.p. and/or cetuximab i.v. administration on the growth of SVCT-2-positive SW620 and SVCT-2-negative HCT116 tumors in nude mice. To this end, cells from each cell line were subcutaneously injected into the mice and allowed to form xenograft tumors, and they were then treated with L-ascorbic acid and/or cetuximab or vehicle. The tumors were treated with 0.5 g/kg L-ascorbic acid and/or 50 mg/kg cetuximab or PBS for 14 days. The growth of the SW620 tumors expressing the SVCT-2 protein was dramatically suppressed in response to the administration of the combination treatment but not compared to the HCT116 tumors that did not express the SVCT-2 protein (Fig. 5A). Next, we investigated the changes in the MAPK pathway, including the RAF protein, using immunohistochemistry in response to the single or the combinatorial treatment. Surprisingly, the combination treatment led to the suppression of the MAPK signaling pathway, including BRAF and CRAF, whereas the single treatment did not induce the suppression of these signaling molecules (Fig. 5B) as shown in Fig. 4. Consistently, the immunohistochemical analysis also demonstrated the suppression of these signaling molecules (Fig. 5C). Thus, the xenograft experiments showed that the combination of L-ascorbic acid and cetuximab significantly decreased the tumor growth of the mutant KRAS-bearing human colon cancer cell xenografts in a manner that was dependent on the expression levels of the SVCT-2 protein.

4. Discussion

Cetuximab, an antibody directed against the epidermal growth factor receptor, remains a major clinical challenge in the treatment of human colon cancers that express mutant KRAS. A variety of mechanisms related to cetuximab resistance have been studied [18,19]. However, the best treatment strategies for colon cancer patients who are resistant to cetuximab have yet to be clearly elucidated. In this study, we demonstrate that L-ascorbic acid bypasses the resistance to cetuximab in human colon cancer in an SVCT-2-dependent manner. These findings may suggest a method to utilize optimal treatment strategies for cetuximab-resistant colon cancer patients.

Recently, our group reported that SVCT-2 plays a role as an indicator for L-ascorbic acid treatment in human breast cancer [14]. Based on this report, we analyzed the inhibitory effects of L-ascorbic acid on mutant KRAS human colon cancer cells that are resistant to cetuximab in a manner that is dependent on the SVCT-2 protein. To this end, the level of the SVCT-2 protein in the human colon cancer cells that express mutant KRAS were first investigated. Interestingly, SVCT-2 was differentially expressed in the tested colon cancer cells (Fig. 1A). Differences in the SVCT-2 expression levels clearly coincided with sensitivities to cetuximab and L-ascorbic acid (Fig. 1B and C). Furthermore, L-ascorbic acid exhibited combinatorial effects together with cetuximab, implying that cetuximab resistance could be surmounted by L-ascorbic acid in a manner dependent on the SVCT-2 protein. Recent studies showing that L-ascorbic acid flows into cells via SVCT-2 but not SVCT-1 support our findings [20]. In addition, cetuximab selectivity is independent of SVCT-2 expression (Supplementary Fig. S1). Thus, L-ascorbic acid may be an effective treatment option to overcome resistance to cetuximab in mutant KRAS human colon cancers that express SVCT-2.

We also addressed the mechanism underlying the cell death induced by L-ascorbic acid and cetuximab. As shown in Fig. 3, both drugs induced apoptotic and necrotic cell death. To further confirm this finding, we analyzed the effects of the Z-VDAD/caspase inhibitor and/or the Nec-1/necrosis inhibitor on cells that were treated with both drugs. Cell death induced by cetuximab and L-ascorbic acid was almost completely blocked by both inhibitors, whereas the treatment with a single inhibitor led to the partial inhibition of cell death, suggesting that L-ascorbic acid and cetuximab induce cell death via two cellular pathways, apoptosis and necrosis.

Based on these results, we evaluated changes in the MAPK signaling pathway (Fig. 4). As cetuximab alone did not decrease MAPK signaling molecules in the mutant KRAS human colon cancer cells [21,22], we focused on changes in the RAF–MEK–ERK pathway after exposure to L-ascorbic acid and cetuximab. Surprisingly, the treatment with both drugs led to decreased phospho-MEK and phospho-ERK as well as phospho-BRAF and phospho-CRAF, which are known to be key molecules for cetuximab resistance [5] in mutant KRAS human colon cancer cells that express the SVCT-2 protein but not in cells that do not express the SVCT-2 protein. Cascade of MAPK signaling activated through oxidative modification of MAP3K [23]. In addition, CRAF binds to the ASK-1 and suppresses its pro-apoptotic activity [24]. According to these reports, we investigated and demonstrated that activation of ASK-1 and p38 pathway was induced by L-ascorbic acid and cetuximab in SVCT-2 expressing cells (Supplementary Figs. 2 and 3). These results suggest that SVCT-2-dependent ROS production induces the activation of the ASK-1 p38 pathway, which modulates cellular apoptosis. To further confirm this finding, we evaluated MAPK signaling molecules in the colon cancer tissues of xenograft animal models (Fig. 5). Changes in these signaling molecules were observed only in the tissues from the mutant KRAS and SVCT-2-positive human colon cancer cell line SW620 but not in tissues from the mutant KRAS and SVCT-2-negative cell line HCT116. Thus, L-ascorbic acid overcomes resistance to cetuximab through the blockade of the MAPK signaling pathway and by initiating the ASK-1–mediated apoptosis pathway. However, this pathway is related to the apoptotic cell death mechanism. Thus, future research will focus on the detailed mechanisms or the unknown molecules that control necrotic cell death after exposure to L-ascorbic acid in mutant KRAS and SVCT-2-positive human colon cancer cells.

In conclusion, differences in the sensitivity to L-ascorbic acid treatment are dependent on SVCT-2 levels in mutant KRAS human colon cancer cells that are resistant to cetuximab. Although we established an important role for the SVCT-2 protein on the anti-cancer effects of L-ascorbic acid in mutant KRAS colon cancer cells, the necrotic cell death of two cellular mechanisms induced by L-ascorbic acid and cetuximab was not addressed. Collectively, our findings highlight the therapeutic potential of L-ascorbic acid for the treatment of cetuximab-resistant mutant KRAS human colon cancer dependent on the SVCT-2 protein.

Conflict of interest

No potential conflicts of interest are disclosed.

Acknowledgments

This study was supported by Grants from the Korea Health 21