

Ascorbyl Stearate Inhibits Cell Proliferation and Tumor Growth in Human Ovarian Carcinoma Cells by Targeting the PI3K/AKT Pathway

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Abstract. Ascorbyl stearate is a lipophilic, vitamin C derivative with antitumorigenic properties. The molecular mechanism(s) underlying the anticarcinogenic effect of this compound have not been well documented. The effect of ascorbyl stearate was studied in a panel of human ovarian epithelial cancer cells. Treatment with ascorbyl stearate caused a dose-dependent inhibition of the cell proliferation. The antiproliferative effect was due to the arrest of cells in the S/G2-M-phase of the cell cycle. Treatment of OVCAR-3 cells with ascorbyl stearate also inhibited PI3K/AKT activity. The presence of a constitutively active AKT protected OVCAR-3 cells from the effects of ascorbyl stearate, suggesting that this nutraceutical targets the PI3K/AKT pathway. The administration of ascorbyl stearate by gavage induced involution of human ovarian carcinoma xenografts in nude mice. These studies indicate that the antiproliferative effect of ascorbyl stearate on ovarian epithelial cancer cells is associated with decreased PI3K/AKT activity, and point toward the PI3K/AKT signaling pathway as a target for this drug.

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Abbreviations: Asc-S, Ascorbyl stearate; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; Myr-AKT, myristoylated AKT.

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Key Words: Ascorbyl stearate, PI3K/AKT, ovarian carcinoma.

Ovarian cancer represents the most lethal gynecological malignancy in the United States with an annual incidence of approximately 23,300 new cases and 13,900 deaths (1). The pathobiology of ovarian epithelial cancer, accounting for about 90% of all ovarian malignant tumors, is unknown. The rate of ovulation, hormonal imbalance and persistent inflammatory conditions are considered to be predisposing factors for the development of this malignancy (2). Ovarian epithelial cancer remains asymptomatic for a long time and is usually detected late in its course, accounting for the high stage and poor survival rate (3, 4). This problem is compounded by the lack of efficient preventive and screening strategies, as well as the frequent development of chemotherapy and radiation resistance. Hence, development of alternative strategies is essential for the management of this disease.

The effects of ascorbyl stearate (Asc-S), an ester of vitamin C, were investigated on a panel of human ovarian carcinoma cells including PA-1, OVCAR-3, A-2780, ES-2, Caov3 and SW626. This compound was chosen because of the considerable epidemiological evidence pointing to the benefits of vitamin C, and of its derivatives, in the prevention and treatment of several types of cancer. Ascorbyl esters are non-toxic, synthetic compounds derived from the esterification of ascorbic acid with a fatty acid (palmitic acid or stearic acid). This reaction confers lipophilic properties to the ascorbate, allowing its passage across biological membranes (5). Ascorbic acid and ascorbyl esters have shown antineoplastic properties against several malignant cell lines (6-10), and have been effective in reducing the incidence of estradiol- and diethylstilbestrol-induced renal tumors in hamster (9). Vitamin C was able to mitigate the mutagenic effects of

alkylating agents *in vivo* (11), and it was found to have a protective role against chemically-induced hepatocellular tumors in rats (12). Other studies have shown the efficacy of vitamin C in enhancing the antineoplastic activity of doxorubicin, cisplatin and paclitaxel in human breast carcinoma cells (13), and in augmenting the therapeutic responses to cisplatin in cervical carcinoma cells (14). Recently, we have shown that Asc-S inhibits cell proliferation and anchorage-independent growth, and induces apoptosis of mouse glioma, human glioblastoma and human pancreatic cancers (15-17).

Since alterations of the mitogenic growth factors, PI3K and AKT, are frequently observed in ovarian cancers (18, 19), whether the treatment of human ovarian cancer cells with Asc-S was able to modify the expression/activation of such molecules was tested. The data obtained demonstrated that Asc-S inhibits cell proliferation of human ovarian carcinoma cells by interfering with cell cycle progression and by modulating cell survival pathways (PI3K/AKT). Using a xenograft nude mouse model, this study also showed that Asc-S causes regression of ovarian carcinomas *in vivo*.

Materials and Methods

Cell culture. The human ovarian carcinoma cell lines PA-1, OVCAR-3, A-2780, ES-2, Caov-3 and SW626 were obtained from the American Type Culture Collection (Rockville, MD, USA). OVCAR-3 and A-2780 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Whittaker MA Bio-products, Walkersville, MD, USA); ES-2 and Caov-3 were maintained in Medium199, SW626 in MCDB 105 (Sigma Chemical Co., St. Louis, MO, USA) and PA-1 cells in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA).

Plasmid transfection. OVCAR-3 cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells/dish. Following overnight incubation, the cells were transfected with 2 µg of DNA per dish, using the Lipofect AMINE Plus (Gibco BRL). The expression construct was characterized by a cytomegalovirus (CMV)-based expression vector encoding Myr-AKT, as previously described (17). After 48 h, 0.6 mg/ml G418 was added to the cells and 3 stable clonal cell lines were established from each transfectant.

Cell growth and viability. The effect of Asc-S on cell growth and viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma Chemical Co.), as described earlier (20). In brief, PA-1, OVCAR-3, A-2780, ES-2, Caov-3 and SW626 human ovarian carcinoma cells, as well as OVCAR-3/AKT and OVCAR-3/pcDNA transfectants, were seeded at 2.0×10^4 viable cells/well in 0.1 ml of DMEM/MEM/M199, or MCDB105 medium supplemented with 10% FBS in 96-well tissue culture plates. After 12 h, the medium was replaced with fresh medium containing 0, 25, 50, 75, 100, 125 or 150 µM of Asc-S and the cells incubated in CO₂ at 37°C for 24 h, with respective DMSO vehicle-treated controls. After 24 h, the MTT dye was

added to the cells and the plates were incubated at 37°C for 3 h. The solubilization reagent DMSO was added and absorbance at 570 nm was determined using a Dynatech MR580 reader with a reference wavelength of 630 nm. The correlation of the absorbance of MTT to cell number was verified in a separate experiment showing a linear relationship between these 2 parameters with a correlation coefficient of 0.98. The decrease in absorbance was considered as loss of cell viability.

Cell growth assay. [³H] Thymidine incorporation in 2×10^4 cells/well in 96-well plates was evaluated as follow: OVCAR-3 cells and OVCAR-3 cells transfected with Myr-AKT (OVCAR-3/AA2) were allowed to attach for 24 h, incubated in growth arrest medium (0.5% FBS) for 24 h, and then incubated for an additional 24 h in fresh medium (10% DMEM) containing 0, 75, 100, 125, 150 or 175 µg of Asc-S. For the last 6 h of incubation, 0.5 µCi/well [³H]methylthymidine (Amersham Pharmacia Biotech) was added. The cells were then harvested onto filters using a cell harvester and counted using a Betaplate liquid scintillation counter (Amersham Pharmacia Biotech). The results were expressed as a percentage of the values obtained for the same cells in the absence of Asc-S. In the time-course experiments, incorporation of [³H]thymidine after treatment with 150 µM of Asc-S was measured at 0, 6, 18, 30 and 42 h.

Cell cycle analysis by flow cytometry. For time-course studies, OVCAR-3 cells growing in DMEM with 10% FBS were synchronized with the double thymidine block method (21), and treated with 150 µM of Asc-S at time-intervals 3, 6, 9, 12, 18 and 24 h. The cells were then trypsinized, washed twice with PBS, centrifuged, resuspended and counted. Asc-S-treated OVCAR-3 cells and DMSO vehicle-treated controls were adjusted to 1×10^6 cells/ml, centrifuged and fixed in 1 ml of ice-cold 70% ethanol overnight at 4°C. The cells were stained with propidium iodide (50 µg/ml) containing RNase (100 U/ml). Samples were run on the FACscan flow cytometer and analyzed using cell fit software.

In vitro kinase assay. Akt kinase and PI3K assays were performed as described previously (17). Briefly, the Akt kinase assay was carried out in the presence of 10 µCi of [^γ-³²P] ATP (Amersham) and 3 µM cold ATP in 30 µl of reaction buffer using histone H2B as substrate. After incubation, the reaction was separated in SDS-PAGE gels. PI3K was immunoprecipitated from the cell lysates with anti-pan-p85 antibody (UBI). After washes, the immunoprecipitates were incubated with reaction buffer containing 10 mM HEPES (pH7.4), 10 mM MgCl₂, 50 µM ATP, 20 µCi [^γ-³²P] ATP and 10 µg L-α-phosphatidylinositol-4,5-phosphate (PI-4,5-P₂, Biomol). The reactions were stopped by adding 100 µl of 1 M HCl. Phospholipids were extracted with 200 µl of CHCl₃/Methanol. The phosphorylated products were separated by TLC as described previously (17). The conversion of PI-4,5-P₂ to PI-3,4,5-P₃ was determined by autoradiography and quantified with Phosphorimager.

Antitumoral efficacy of Asc-S in nude mice. The antitumoral efficacy of Asc-S was tested *in vivo* using a human ovarian carcinoma xenograft nude mouse (C57BL/6) model with prior Institutional Animal Care and Use Committee approval (IACUC #1593). Eight-week-old female nude mice were injected subcutaneously above the hind leg with 5×10^5 cells of OVCAR-3

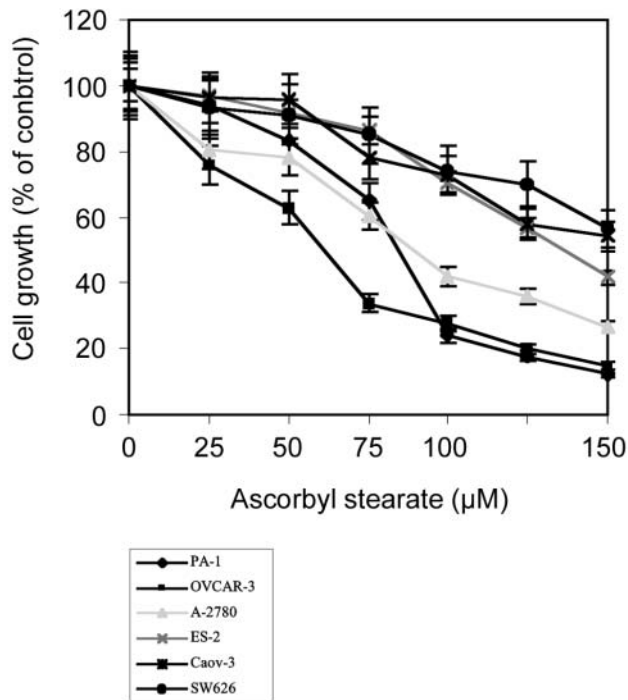


Figure 1. Asc-S inhibits ovarian cancer cell proliferation. Exponentially-growing human ovarian carcinoma cells were exposed to the indicated amounts of Asc-S for 24 h. The cell growth and viability of cells was determined by MTT assay. The results are expressed as a percentage of the control (untreated cells). The values are mean $\pm$ SD of 3 individual experiments.

and OVCAR-3 transfected with Myr-AKT suspended in sterile PBS. All mice developed subcutaneous tumors after 7 days. When the tumors had reached about 3.2 mm in diameter, the animals were divided in 2 groups of 6 mice each, and each mouse was injected with OVCAR-3 cells on the right side and with OVCAR-3/Myr-AKT on the left side. One group was treated with Asc-S at a dose of 250 mg/kg/day, dissolved in 0.05 ml DMSO and with PBS added to a final volume of 0.25 ml, while the other group received the vehicle alone (DMSO and PBS). The treatment was continued for 27 days. Tumor dimensions were measured every 3 days with microcalipers. At the end of the treatment, the mice were euthanized and tissue samples from the site of injection were processed for histological examination. The mean reductions in the tumor sizes was calculated for each group, and at each time-point, and compared using the independent two sample Student's *t*-test at the two-sided significance level of 0.05. The data were also analyzed with ANOVA.

Results

Asc-S inhibits ovarian cancer cell proliferation by delaying cell cycle progression. Incubation of the human ovarian cancer

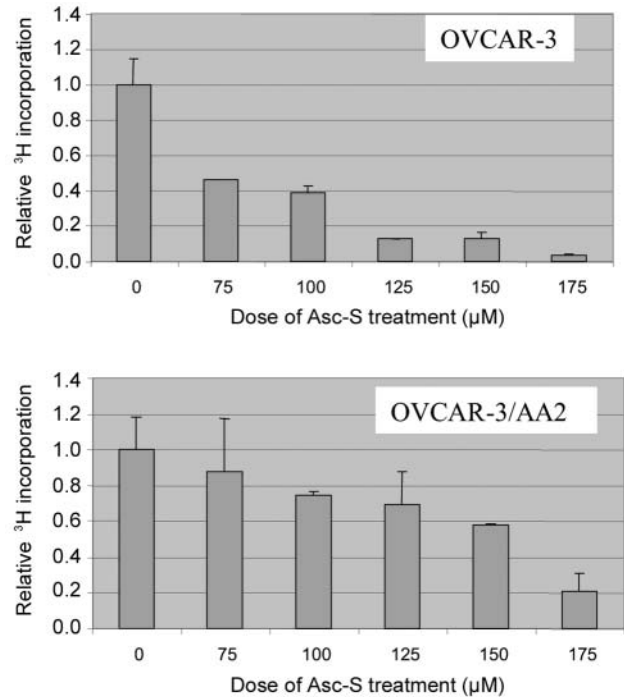


Figure 2. [³H]-Thymidine incorporation. Exponentially-growing OVCAR-3 cells and OVCAR-3 cells transfected with myr-AKT (OVCAR-3/AA2) cells were exposed to DMEM medium containing different concentrations of Asc-S (0, 75, 100, 125, 150, 175 μM) for 24 h. During the last 6 h of incubation, 0.5 mCi/well of [³H]-methylthymidine was added. The cells, harvested onto filters, were subjected to a beta plate liquid scintillation counter. The results are expressed as relative thymidine incorporation compared to the same cells in the absence of Asc-S. The results are mean $\pm$ SD of 3 individual experiments.

cells PA-1, OVCAR-3, A-2780, ES-2, Caov-3 and SW626 with different concentrations of Asc-S (0, 25, 50, 75, 100, 125 or 150 μM) resulted in significant inhibition of cell growth. The reduction in cell growth was in the range of 46 to 88%, in different cell lines using 150 μM of Asc-S, compared to the vehicle (DMSO)-treated cells. Among the ovarian cancer cells tested, PA-1 and OVCAR-3 were the most sensitive (Figure 1). The decrease in cell proliferation was corroborated in OVCAR-3 cells by a dose-dependent incorporation of [³H]thymidine following exposure to Asc-S (0, 25, 50, 75, 100, 125, 150 and 175 mM) for 24 h (Figure 2).

To investigate the effect of Asc-S on the cell cycle, cell cycle analysis was performed employing OVCAR-3 cells. Asc-S treatment (150 μM) resulted in a significant increase in the percentage of cells arrested at the G2/M-phase of the cell cycle. Thus 35%, 55%, and 67% of Asc-S-treated cells were arrested in the G2/M-phase at 9, 12 and 18 h, respectively. In addition, the progression of cells from the S-phase to G2-M was slow at the initial time-points (3-9 h), followed by arrest at the G2/M-phase (Figure 3).

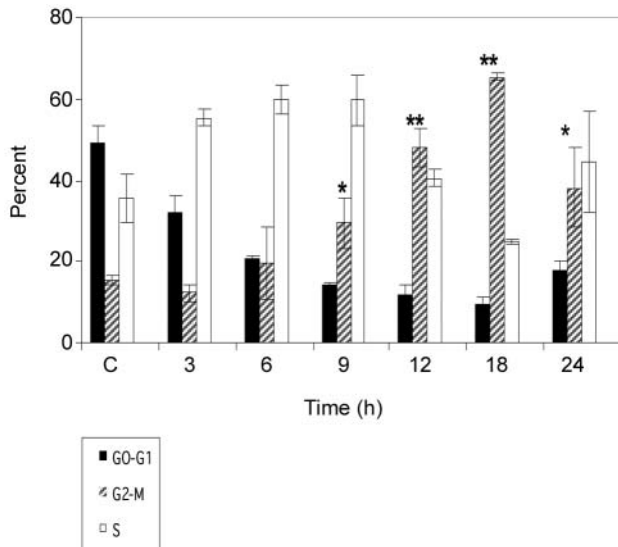


Figure 3. Effect of Asc-S on cell cycle distribution. OVCAR-3 cells were synchronized by double thymidine block method. The synchronized cells were exposed to 150 μ M of Asc-S for the indicated times and analyzed by flow cytometry. The data indicate the percentage of cells in each phase of the cell cycle. The results are expressed as mean $\pm$ SD of 3 individual experiments.

OVCAR-3 cells with a constitutively active AKT are resistant to the antiproliferative effect of Asc-S. AKT is often amplified or constitutively activated in human ovarian cancer. Therefore, OVCAR-3 cells were transfected with Myr-AKT (OVCAR-3/AKT) to evaluate whether the presence of a constitutively active AKT would modify the sensitivity of these cells to Asc-S treatment. The inhibitory effect of Asc-S on [3 H] thymidine incorporation was found to be considerably minimized in OVCAR-3/AKT transfectants (Figure 4).

Asc-S inhibits AKT and PI3 kinase activities. Previous studies showed that AKT activation by mitogenic growth factors is mediated by PI3K and that alterations of PI3K and AKT are frequently observed in ovarian cancer. Therefore, whether Asc-S inhibits endogenous AKT activation was investigated. OVCAR-3, SW626, ES-2, A2780, CaOv3 and PA-1 cells treated with 150 μ M Asc-S for 24 h showed decreased phospho-AKT expression as compared to the DMSO vehicle-treated controls. The SW626 cells showed no significant expression of phospho-Akt even in the absence of Asc-S treatment. Interestingly, SW626 was the least sensitive cell line to Asc-S (Figure 5A). A time-course effect of Asc-S on OVCAR-3 cells revealed that the decrease in phospho-AKT can first be detected at 12 h, in the presence of an unchanged level of total AKT protein (Figure 5B).

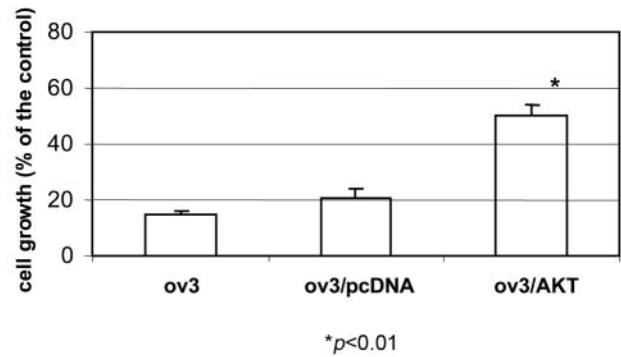


Figure 4. The antiproliferative effect of Asc-S is inhibited by the expression of constitutively active AKT. OVCAR-3 cells were stably transfected with Myr-AKT. The transfected cells were treated with 150 μ M of Asc-S for 24 h. The cell growth/viability was determined by MTT assay. The results are expressed as mean $\pm$ SD of 3 experiments.

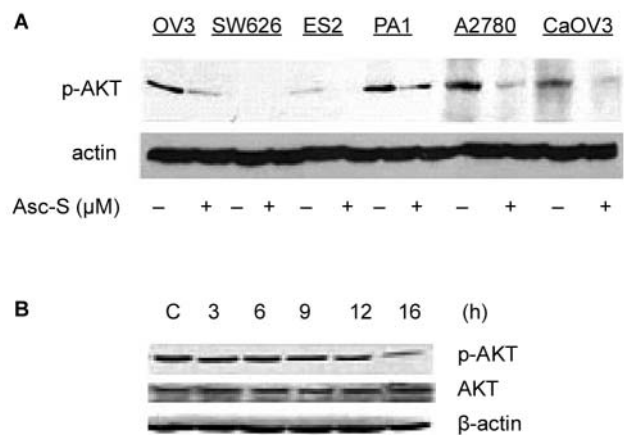


Figure 5. Asc-S inhibits the phosphorylation of endogenous AKT. (A) Asc-S inhibits phosphorylation levels of AKT in different ovarian carcinoma cell lines. The cells were treated with 150 μ M Asc-S for 24 h and immunoblotted with anti-phospho-AKT-S473 antibody. (B) Following the treatment with 150 μ M Asc-S for the indicated time, parental OVCAR-3 cell lysates were immunoprecipitated with anti-AKT antibody and the immunoprecipitates were immunoblotted with anti-phospho-AKT-S473 (top), -total Akt (middle) and β -actin (bottom) antibodies. C indicates untreated cells (time 0).

Next the question of whether PI3K activity is inhibited by Asc-S was investigated. Following treatment with or without Asc-S and IGF1, OVCAR-3 cells were lysed and immunoprecipitated with anti-pan-p85 antibody. The immunoprecipitates were subjected to an *in vitro* kinase assay using PI-4,5-P2 as substrate. As shown in Figure 6A, IGF1-induced PI3K activity was significantly suppressed by Asc-S, suggesting that Asc-S targets the PI3K/AKT pathway. To further investigate whether Asc-S is a direct inhibitor of PI3K

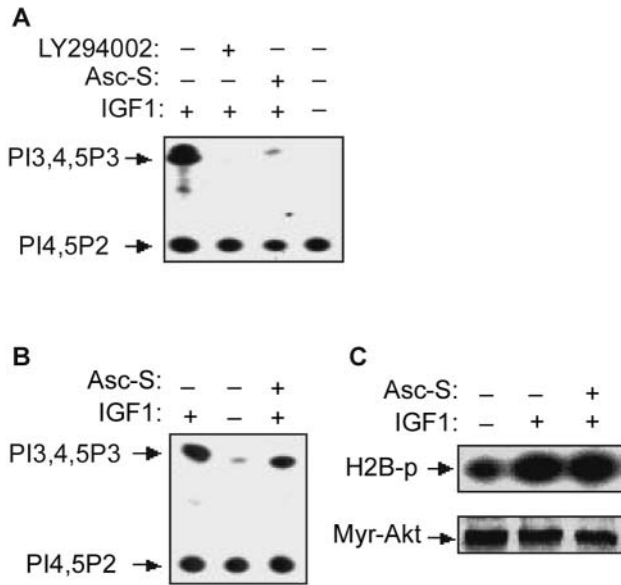


Figure 6. Asc-S targets the PI3K pathway but is not a direct inhibitor of PI3K and AKT. (A) *In vitro* PI3K kinase assay. OVCAR-3 cells were serum-starved and treated with Asc-S for 16 h prior to IGF1 stimulation for 15 min. The cells were lysed and immunoprecipitated with anti-pan-p85 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay using PI-4,5-P2 as substrate. (B and C) *In vivo* kinase assay. OVCAR-3 cells were serum-starved and stimulated with IGF1 for 15 min. The PI3K and AKT were immunoprecipitated with anti-pan-p85 and AKT antibodies. PI3K (B) and AKT (C) immunoprecipitates were assayed in kinase reaction buffer containing Asc-S (50 μ M). PI4, GP2 and histone H2B were used as substrate, respectively.

and AKT, OVCAR-3 cells were serum-starved overnight and stimulated with or without IGF1 for 15 min. Immunoprecipitations were carried out with anti-pan-p85 and anti-AKT antibody. The immunoprecipitates were subjected to *in vivo* kinase assays in a kinase buffer containing 100 μ M Asc-S. Repeated experiments showed that Asc-S was unable to directly inhibit PI3K and AKT activities (Figure 6B and 6C) suggesting that Asc-S targets molecules upstream of PI3K.

Antitumorigenic efficacy of Asc-S in nude mice. Nude mice bearing OVCAR-3 tumors treated by gavage with Asc-S showed a significant reduction in tumor size compared with the control group. Both groups of mice had tumors of the same diameter (control = 3.19 mm; Asc-S-treated = 3.2 mm) at baseline. Histological examination revealed poorly-differentiated ovarian carcinomas. With the passage of time, the differences in tumor diameter became larger, the maximum difference being observed at day 27 after injection (8.9 mm control vs. 5.5 mm Asc-S-treated; $p=0.007$). OVCAR3/Myr-AKT tumors were found to be insensitive to Asc-S treatment (Figure 7).

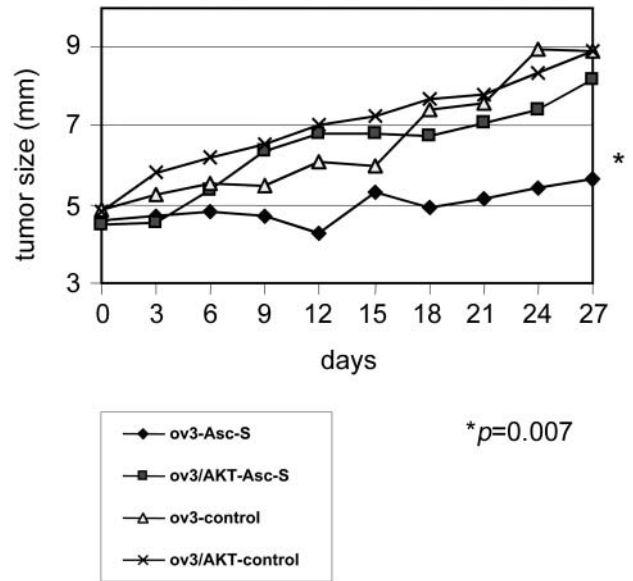


Figure 7. Asc-S inhibits tumor growth. OVCAR-3 and OVCAR-3/AKT cells were subcutaneously injected into 12 nude mice. After the tumors had reached about 3.2 mm in diameter, the mice were randomly divided into 2 groups of 6 mice each, and treated as described in "Materials and Methods". In the "treatment group" the animals were administered daily by gavage 250 mg/kg Asc-S dissolved in 0.025 DMSO and distributed in 0.25 ml PBS. Ov3 control and ov3-AKT-control = OVCAR-3 and OVCAR-3/Myr-AKT tumors treated with vehicle (DMSO + PBS) alone.

Discussion

The results of this study showed that exposure of ovarian epithelial cancer cells to Asc-S inhibited cell proliferation and tumorigenesis and was associated with inhibition of PI3K/AKT activities.

The effect of Asc-S in ovarian epithelial cancer is a novel finding, although the antitumoral properties of vitamin C and its derivatives, as well as their adjuvant action in combination with other antitumoral agents, are well known (2-15, 20, 22). However, there have been controversial results concerning such properties (23, 24), possibly reflecting the lipophobic nature of the ascorbic acid. In this study we focused on the effects of Asc-S, a vitamin C derivative obtained by esterification of ascorbic acid with stearic acid, on ovarian epithelial cancer. This particular vitamin C derivative was chosen because the presence of the fatty acid chain makes the compound lipophilic, thus allowing it to easily cross biological barriers (5, 25), much enhancing the biological effects of ascorbic acid. We have previously reported that the antiproliferative effect of Asc-S on glioblastoma multiforme cells was 68-fold more potent than that of sodium ascorbate; Asc-S-induced growth inhibition has

also been observed following treatment of human pancreatic carcinoma cells (15, 16, 20, 22).

In this study, it was shown that the antiproliferative effect of Asc-S on human ovarian carcinoma cells is due to their inability to complete the cell cycle, and to their accumulation in the late S/G2/M-phase of the cell cycle (G2/M checkpoint). This finding is in agreement with a previous report showing that dehydroascorbic acid, the oxidized form of ascorbic acid, arrests cells in the late G2/M-phase during oxidative stress (26).

The AKT family members, AKT 1, 2, and 3, have been shown to be either amplified or constitutively activated in human tumors such as breast, pancreatic, ovarian, prostatic and gastric carcinomas (18, 19, 27, 28). When tested, all of the ovarian cancer cell lines under study, with the exception of SW626 (the least sensitive to Asc-S exposure), displayed high levels of activated AKT (27). In this report, we showed that Asc-S treatment reduced AKT and PI3K activities in OVCAR-3 cells, and that the ectopic expression of Myr-AKT rendered them insensitive to Asc-S.

Our data also showed an antitumorigenic effect of Asc-S *in vivo* using a human ovarian carcinoma xenograft nude mouse (C57BL/6) model. This finding is consistent with numerous reports showing the antitumorigenic effect of AKT-mRNA antisense strategies in different tumor types (29, 27). In our study, the significant antitumorigenic effect was achieved on administering the Asc-S by gavage, a non-invasive administration route. Asc-S and its breakdown products (ascorbate and stearic acid) are virtually non-toxic to biological systems ($LD_{50} = 25$ g/kg of Asc-S for mice), and have been reported to have anticarcinogenic properties in different tumor types (7, 15, 16, 20, 22). Ascorbyl radicals, produced by oxidation of Asc-S, are reported to induce apoptosis in renal cell carcinoma and glioblastoma tumor cells (30, 31). In addition, it was here demonstrated that modulation of PI3K/AKT by Asc-S may account for the effects of this compound on the proliferation and tumorigenesis of human ovarian epithelial cancer cells.

In conclusion, Asc-S, a lipophilic derivative of vitamin C, has antiproliferative effects on ovarian carcinoma cells that are associated with decreased PI3K/AKT activity, suggesting that this compound may represent a potential nutraceutical for the treatment of human ovarian epithelial cancer.

Acknowledgements

We thank the Molecular Biology, Molecular Imaging, Flow Cytometry and Pathology Core Facilities of the Moffitt Cancer Center and Research Institute, U.S.A.

This work was supported by the Department of Defense Project Grant, DAMD 17-02-1-0670 (D.C., S.V.N. and J.Q.C.) and by National Cancer Institute grants CA77935 and CA89242 (J.Q.C.). We also thank Dr. Roberto F. Nicosia for this expert review of this article.

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Received August 22, 2005

Accepted October 17, 2005