p53 Enhances Ascorbyl Stearate-induced G2/M Arrest of Human Ovarian Cancer Cells

K. AKHILENDER NAIDU[1,5*], QUAN FANG[3*], KAMATHAM A. NAIDU[4], JIN Q. CHENG[2,3], SANTO V. NICOSIA[1,3] and DOMENICO COPPOLA[1,3]

Divisions of [1]Anatomic Pathology and [2]Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida; [3]Department of Pathology, University of South Florida, College of Medicine, Tampa, Florida; [4]Baylor-Sammons Cancer Center, Dallas, Texas, USA; [5]Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570013, India

Abstract. *Background: Ascorbyl stearate (Asc-S) is a synthetic ester of ascorbic acid that has been shown to significantly reduce the mutagenic effects of alkylating agents and hepatocarcinogenesis in vivo. We have previously demonstrated that Asc-S inhibits ovarian carcinoma cell proliferation through modulation of the cell cycle. This study was designed to further elucidate the mechanisms underlying such regulation. Materials and Methods: Wild type p53-expressing cell lines (Ov2008 and C13) were used to evaluate the contributions of p53 to Asc-S-induced G2/M arrest. Cell cycle analysis was performed by flow cytometry. Variation of p53, p21, and GADD45 was evaluated by Western blot and RT-PCR. Knockdown of endogenous p53 was achieved by si-RNA. Results: The expression of p53 downstream genes, p21 and GADD45 was upregulated whereas 14-3-3σ was unaffected. Phosphorylation of Cdc2 at residue tyrosine-15 was also induced by Asc-S treatment. However, pSilencer–p53–siRNA only partially rescued the Asc-S induced G2/M arrest. Conclusion: These data show that the anti-proliferative activity of Asc-S on ovarian cancer cells is due in part to G2/M arrest modulated by a p53-dependent pathway.*

*Both authors contributed equally to this work (QF recently moved to the Department of Pharmacology at the Medical University of South Carolina, Charleston, SC, USA).

Correspondence to: Domenico Coppola, MD, Department of Interdisciplinary Oncology – Division of Anatomic Pathology, Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, 12902 Magnolia Drive, Tampa, FL 33612, U.S.A. Tel: +18137453275, Fax: +18137451708, e-mail: domenico.coppola@moffitt.org

Key Words: Ascorbyl stearate, cell cycle, G2/M arrest, ovarian carcinoma.

Despite several treatment options, ovarian carcinoma remains the fifth most common cause of cancer-related mortality in women (1). One major problem is that this form of human cancer is clinically silent for a long time and, when diagnosed, is usually widespread and has a poor survival rate (2). The second problem is that ovarian carcinomas are prone to develop multidrug resistance. After treatment with various regimens, including cisplatin, taxol-based regimens and doxorubicin, many patients experience relapse with chemoresistant disease. Thus, there is a need for new and more effective anticancer drugs.

Ascorbic acid and its derivates are well-known antioxidants and their anticarcinogenic activity is currently being investigated in a number of malignant cell lines (3-5). Among ascorbic acid derivatives, fatty acid esters of ascorbic acid, such as ascorbyl palmitate and ascorbyl stearate, have attracted considerable interest as anticancer compounds. Because of their lipophilic nature, they can easily cross the cell membrane and the blood-brain barrier (6). Ascorbic acid and ascorbyl esters have been shown to inhibit the proliferation of mouse glioma, human brain tumor cells and renal carcinoma cells (7-10). Among the esters, ascorbyl stearate (Asc-S) was found to be more potent than sodium ascorbate in inhibiting the proliferation of human glioblastoma cells (11).

We have previously reported that the antiproliferative effect of Asc-S in a variety of tumors is due to interference with cell cycle progression and induction of programmed cell death (11-12). While the facilitation of apoptosis seems to involve inhibition of the PI3K/AKT pathway, the molecular mechanisms underlying the induction of G2/M arrest have not yet been well-elucidated.

The goal of this study was to evaluate the role of p53 in Asc-S induced G2/M arrest. We focused on p53 because of its known pivotal role in the regulation of the cell cycle, apoptosis and DNA repair (13-15). p53 is able to block cell cycle progression by inducing p21. In particular, p21 protein

inhibits cyclin dependent kinase 4 (CDK4) complexed with cyclin D, cyclin dependent kinase 2 (CDK2) complexed with cyclins A and E, as well as Cdc2, complexed with cyclin B, arresting the cell cycle (16). Furthermore, the Cdc2-cyclin B kinase is pivotal in regulating G2/M transition. Subsequent to cyclin binding, Cdc2 may become phosphorylated on three regulatory sites: threonine-14, tyrosine-15 and threonine-161. Phosphorylation of threonine-14 and/or tyrosine-15 near the N-terminus activates Cdc2, whereas phosphorylation of threonin-161 near the C-terminus has inhibitory function (15-16). Both Cdc25C and p53 can activate the Cdc2-cyclin B complex resulting in G2/M arrest. In late G2, the Cdc25C phosphatase dephosphorylates Cdc2 on threonine-14 and tyrosine-15, leading to activation of Cdc2/cyclin B complexes. p53 has been reported to play a regulatory role during the G2/M transition, particularly after DNA damage. Because of its prominent role in the regulation of cell cycle, we hypothesized that p53 may be a target of Asc-S and thus be responsible for the observed G2/M arrest.

We used wild-type p53-expressing cell lines (Ov2008 and C13) to evaluate effects of Asc-S-induced p53 variations on the cell cycle and on the expression of cell cycle-related proteins p21 and GADD45. We also evaluated the effect of p53 blockage by si-RNA on the tumor cell sensitivity to Asc-S.

Materials and Methods

Cell culture and treatment. The human ovarian cancer cell lines OV2008 and C13 were cultured in RPMI-1640 medium supplement with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD, USA) at 37˚C in 5% CO_2. Normal human ovarian surface epithelial cells were immortalized with an SV40 expression plasmid (IMCC) and grown in medium 199 with 10% fetal bovine serum (FBS). Cells were plated at least 2 days before drug treatment. Asc-S, supplied by Dr. Naidu (Baylor College of Medicine, Texas, USA), was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA). After treatment with Asc-S, at the doses listed for each experiment, cells were gently washed, incubated in fresh media at 37˚C, and harvested at subconfluent conditions at various time points.

Cell growth and viability. The effect of Asc-S on cell growth and viability of human ovarian cancer cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MD, USA). In brief, wild-type p53 expressing OV2008 cell line, and its derived C13 cisplatin resistant cell line were seeded at $2.0x10^4$ viable cells/well in 0.1 ml of medium 199 supplemented with 10% FBS in 96-well tissue culture plates. After 12 h, the medium was replaced with fresh medium containing 0, 50, 100, 150 and 200 μM of Asc-S and the cells incubated in CO_2 at 37˚C for 24 h with respective DMSO vehicle treated controls. After 24 h, 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 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 two parameters with a correlation coefficient of 0.98. The decrease in absorbance was considered as loss of cell viability. The statistical analysis was performed by ANOVA.

Cell cycle analysis by flow cytometry. At each time point, cells attached to the culture dishes were trypsinized. These cells were washed in phosphate-buffered saline (PBS), fixed in 90% (v/v) ethanol overnight, and treated with 100 U/ml RNase A (Sigma Chemical Co.). The nuclei were stained with 50 μg/ml propidium iodide (Sigma Chemical Co.) before the DNA content was measured using FACSCalicur (Becton Dickinson, Inc., Franklin Lakes, NJ, USA). The ModFit LT V2. 0 software package (Verity Software House, Inc., Sunnyvale, CA, USA) was used to analyze the data.

Antibodies and immunological analysis. Monoclonal antibodies specific for p53 (BP53-12), β-actin (Sigma-Aldrich Co., St. Louis, MO, USA), p53-Ser15 (16G8) and Cdc2-Tyr15 (Cell Signaling Technology, Inc., Danvers, MA, USA); p21 (F-5) and Cdc-2 p34 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. Polyclonal antibodies specific for GADD45 (c-20), Cdc25C (C-20), p-Cdc25C-Ser216 (sc-12354), 14-3-3σ (N-14) were obtained from Santa Cruz Biotech. Cells were lysed in ice-cold buffer containing 0.5% NP40 (v/v) in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, protease inhibitor (2 μg/ml aprotinin, leupeptin, 50 μg/ml antipain, and 1 mM phenylmethylsulfonyl fluoride), 1 mM Na_3VO_4, and 1 mM NaF. Lysates were incubated for 30 min on ice before centrifugation at 14,000 rpm for 5 min at 4˚C. Proteins in the supernatant were denatured by boiling for 5 min in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes for immunoblotting. The membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (10 mM pH 7. 6 Tris-HCl, 150 mM NaCl, 0. 5% Tween) and incubated with the indicated antibodies. Bound antibodies were detected with HRP-conjugated secondary antibodies using Electrochemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

Semi-quantitative reverse transcription-PCR (RT-PCR). Total RNA was purified from cells using a guanidinium isothiocyanate method (TrioZol, Gibco, Invitrogen Corporation, Carlsbad, CA, USA). A 1 μg of total RNA was employed from cDNA synthesis using the Superscript Preamplification System and an oligo(dT) primer (Gibco, Invitrogen Corporation, Carlsbad, CA, USA); 10% of the first-strand reaction was then used for semi-quantitative PCR. Before proceeding to first-strand cDNA synthesis, any residual genomic DNA was removed from the samples by incubation with RNase-free DNase I; control reactions were performed in order to ensure complete removal of DNA. The primers used were as follows: actin: (sense) 5'-CAGGT CATCACCATTGGCAAT GAGC-3', (antisense) 5'-GATGTCCA CTCACACTTCATGA-3'; GADD45: (sense) 5'-ATGACTTT GGAGGAATTCTCGG-3', (antisense) 5'-TCACCG TTCGGGGA GATTAATC-3'; p21: (sense): 5'-CAAGCTCTACCTTCCC ACGG-3'; (antisense): 5'-GCCAGGGTAT GTACATGA GG-3'.

The amplification parameters were as follows: 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 1 min, respectively. Standard PCR reactions were performed with 30 cycles. The PCR products were electrophoresed in 2% agarose gels, which were then illuminated with UV light and photographed.

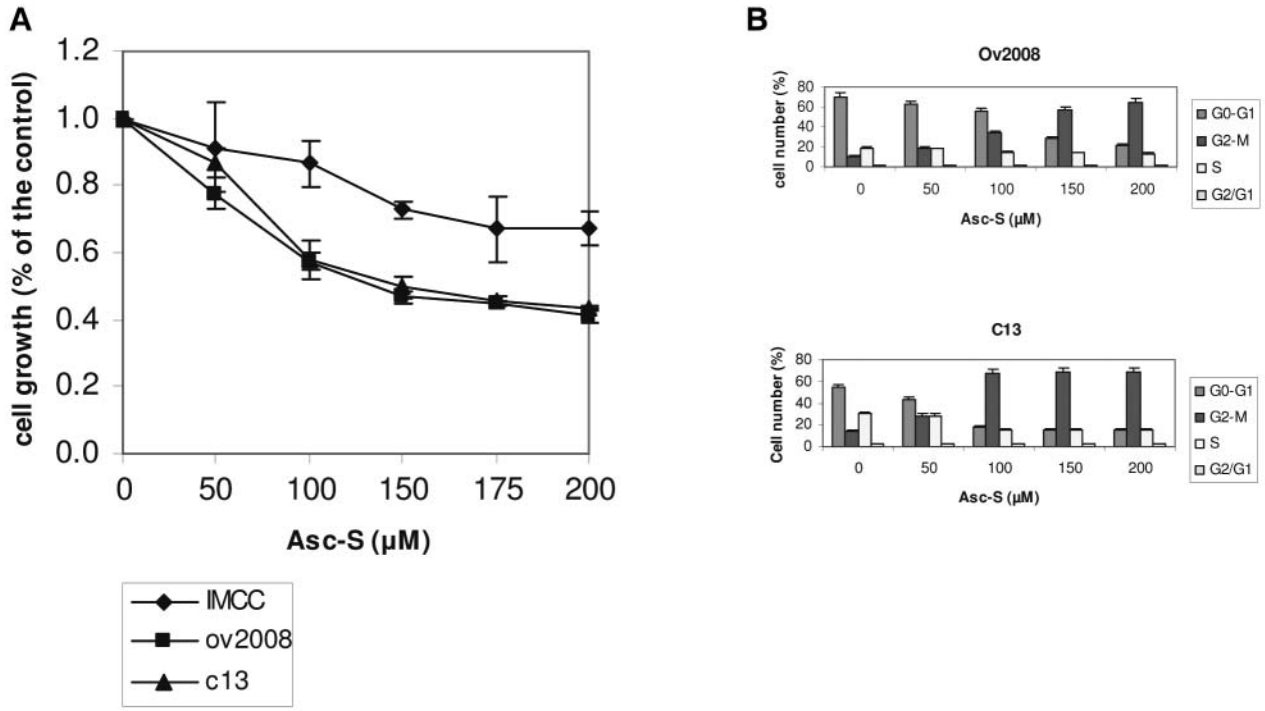

Figure 1. *A) Dose dependent growth inhibition of ovarian cancer cells and immortalized human ovarian epithelial cells following exposure to Asc-S for 24 h. Exponentially growing human ovarian carcinoma cells were exposed to increasing concentrations of Asc-S for 24 hr. The cell growth and viability of cells was determined by MTT assay. Cell numbers are expressed as a percentage of that of controls (untreated cells). The values are the mean of three individual experiments. B) Effect of Asc-S on the cell cycle distribution of ovarian cancer cells. The synchronized cells, after Asc-S treatment for 24 hr, were analyzed by flow cytometry. The data indicate the percentage of cell in each phase of cell cycle. The results are expressed as mean±SD of three individual experiments.*

Nuclear extract preparation and electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from 10^6 Ov2008 and C13 cells, before and after exposure to 150 μM of Asc-S, by high-salt extraction into 30-70 μl buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain) producing approximately 30-90 μg of total protein. EMSAs were conducted with the LightShift Chemiluminescent EMSA kit (Pierce). The cell nuclear extracts were incubated with biotin labeled oligonucleotide probes containing a p53 binding site in the *p21* gene promoter (sense strand, 5'- -3') and a p53 binding site in the third intron of the *GADD45* gene. The probes were labeled using a biotin 3' end DNA labeling kit (Pierce). Five μg of total extracted protein were used for each lane. Protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE), transferred to nylon membranes (Amersham Biosciences, Piscataway, NJ, USA) and then detected by chemiluminescence.

Knockdown of endogenous p53 by siRNA. siRNA-mediated knockdown of endogenous p53 was performed essentially as described by Brummelkamp *et al.* (17). A 64-base pair double-stranded oligonucleotide was synthesized that included a p53 target sequence (GCAUGAACCGGAGGCCCAU), an antisense sequence and a loop, and the construct was cloned into pSilencer vector (Ambion, Applied Biosystems, Austin, TX, USA). The green fluorescent protein (GFP) sequence was used as a non-specific control. The siRNA plasmids were transfected into Ov2008 cells using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA). Cells were isolated 48 h after transfection and stable clones were selected by exposure to 500 μg/ml G418.

Results

The antiproliferation effect of Asc-S is due to G2/M arrest in human ovarian cancer cells. As shown in Figure 1A, the dose response curve indicates that Asc-S inhibited cell growth in both OV2008 and C13 lines but to a much less extent in SV40 immortalized normal human ovarian surface epithelial cells (IMCC). The difference in cell growth between IMCC and OV2008, and between IMCC and C13 was statistically significant (*p*-value 0. 001 and 0. 002, respectively). To assess the mechanism by which Asc-S rescues MTT reduction, we performed cell cycle analysis. Induction of G2/M arrest in Ov2008 cells was found to be highly sensitive and dose-dependent, and exposure to a concentration of 200 μM (Asc-S for 24 h resulted in almost complete cell cycle arrest. Unexpectedly, C13 cells were even more sensitive, showing

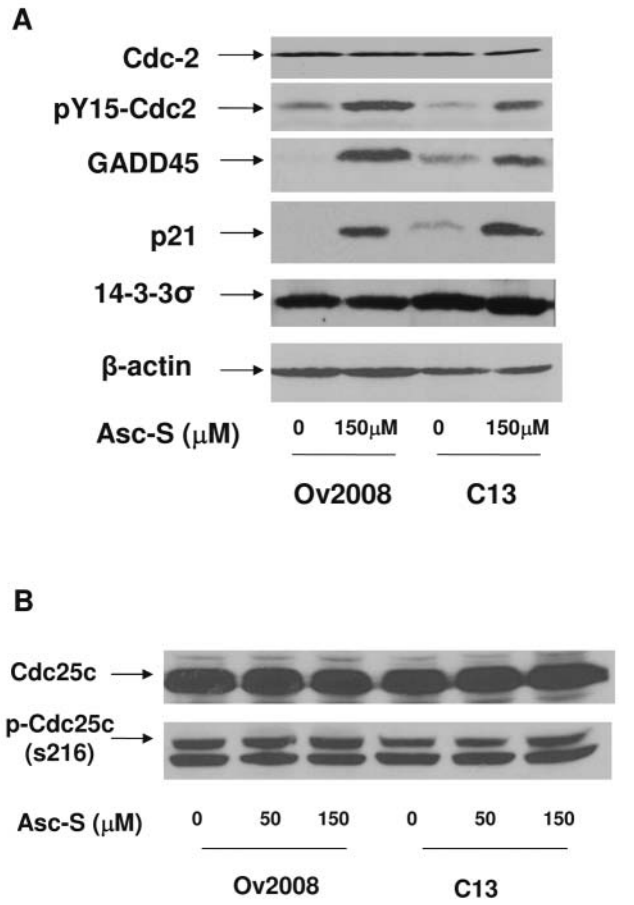

Figure 2. *A) Asc-S induced protein expression of phospho-Cdc2, p21 and GADD45, but not total Cdc2 and 14-3-3σ in both Ov2008 and C13 cells. Western blot was performed to evaluate the expression of target protein. β-actin was used as internal control. B) Neither expression of Cdc25C nor phospho-Cdc25C on ser216 were affected by Asc-S treatment in Ov2008 and C13 cells.*

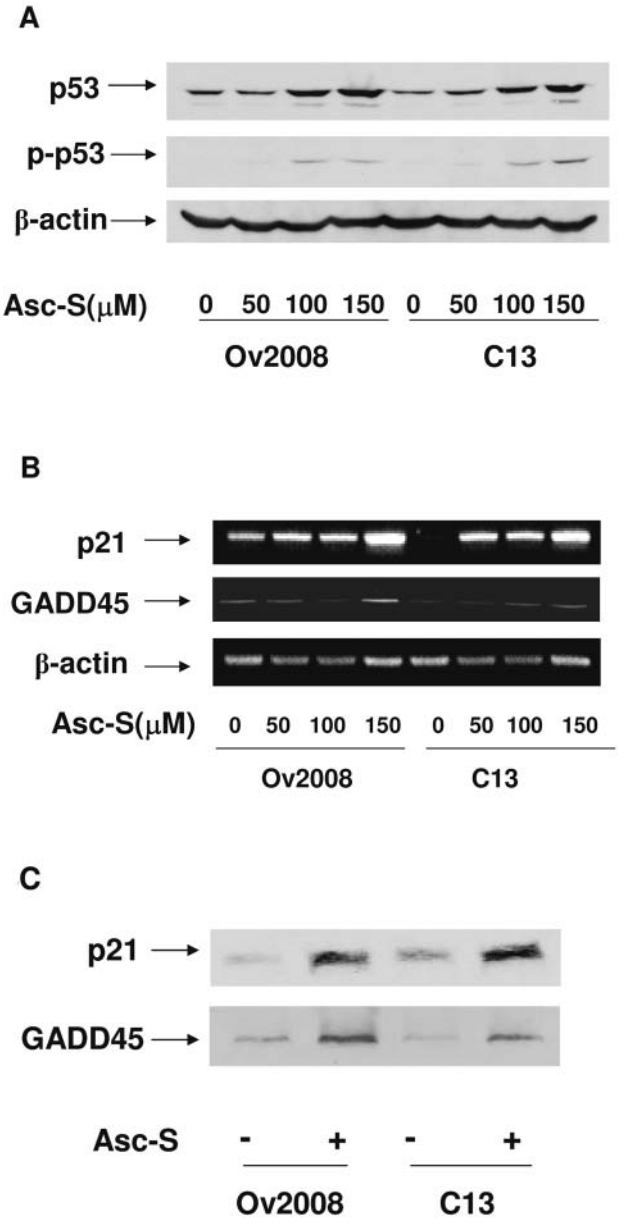

Figure 3. *A. Asc-S induced p53 and phospho-p53 expression in a dose-dependent manner. The protein levels of p53 and p-Ser15-p53 increased after exposure to 0, 50, 100 and 150 μM of Asc-S. Both total and phospho-p53 are highest at 150 μM. B) Asc-S increased the mRNA levels of p21 and GADD45 in a dose-dependent manner. PCR was performed to quantify the expression of target genes using β-actin as an internal control. C) Asc-S induced p53 activated transcription of p21 and GADD45 genes through direct physical interaction with the p21 promoter.*

almost 65% G2/M arrest at 100 μM Asc-S (Figure 1B). A time-course experiment using Ov2008 cells showed that 24 hours after treatment, the majority of cells (80%) were arrested in the G2/M phase (data not shown).

p53 activates Cdc2, resulting in G2/M arrest. To define the mechanism of Asc-S induced G2/M arrest, the protein level and phosphorylation of Cdc2, Cdc25C and p53 were investigated by immunoblotting. Consistent with the G2/M arrest data, Cdc2-p-tyrosine 15 increased after Asc-S treatment, whereas the total Cdc2 protein level did not change (Figure 2A). Cdc25C and its serine 216 phosphatase activity were not altered by Asc-S treatment (Figure 2B). However, both total p53 and p53-p-serine 15 were upregulated (Figure 3A), demonstrating that Cdc2 and p53 play an important role in the Asc-S induced G2/M arrest.

The transcriptional targets of p53, p21 WAF1/CIP1 and GADD45 are up-regulated by Asc-S treatment. Western blot analysis showed that both p21 and GADD45 protein increased following treatment with Asc-S (Figure 2A).

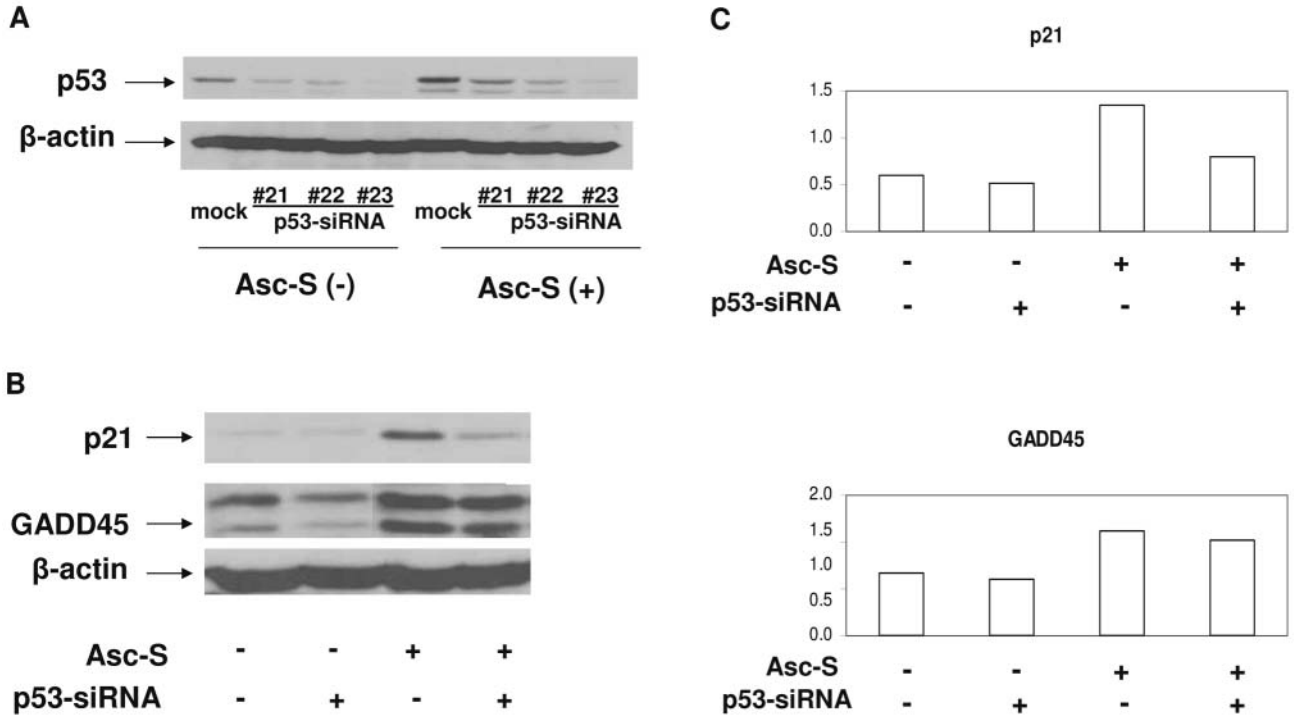

Figure 4. *Inhibition of p53 protein expression by siRNA blocks p21 (A) and GADD45 expression (B). Densitometric analyses of Western blots are shown in C.*

Semi-quantitative, RT-PCR was performed to confirm that the upregulation of GADD45 and p21 was caused by an increase in mRNA. As shown in Figure 3B, treatment of Ov2008 and C13 cells with Asc-S resulted in a concentration-dependent increase in GADD45 and p21 mRNA levels. These results indicate that Asc-S induced production of p21 and GADD45 protein *via* upregulation of mRNA transcription.

To confirm that p53 interacts directly with p21 and GADD45 EMSAs were performed. As shown in Figure 3C, DNA-protein complexes were detected, indicating that the ability of p53 protein binding to its targets was enhanced by the Asc-S treatment.

Blockage of p53 results in inhibition of Asc-S induced p21 and GADD45 and rescue of G2/M arrest. To verify that p53 is required for Asc-S induced upregulation of p21 and GADD45 in Ov2008 cells, we used siRNA to knockdown p53. pSilencer–p53–siRNA plasmid was transfected into OV2008 cells and three stable clones were selected by G418. p53 protein levels in the clones were determined by Western blot. As shown in Figure 4A, p53 protein levels in the clones were markedly diminished as compared to control cells. Following Asc-S treatment (150 μM/24 hours), p53 protein expression increased 3- to 5-fold in control cells, whereas p53 protein was detected only at low levels in the p53-siRNA clones. We then tested GADD45 and p21 proteins expression in the clones and these were both reduced (Figure 4B and C). However, after exposure of p53-siRNA clones to Asc-S, GADD45 increased 4- to 5-fold, demonstrating that Asc-S was still able to arrest these cells in G2/M and to induce GADD45 independently of p53 status.

Cell cycle analysis studies by flow cytometry were performed to further evaluate the role of p53 in Asc-S induced G2/M arrest. Consistent with prior studies demonstrating that Asc-S-induces G2/M arrest in Ov2008, we found that 50-55% of OV2008 cells transfected with pSilencer–p53–siRNA plasmid were arrested in the G2/M phase after exposure to Asc-S (150 μM/24 h), as compared to 70% of the cells transfected with the control plasmid (Figure 5). This result indicates that blockage of p53 only partially rescued Asc-S-induced G2/M arrest.

Discussion

Our data shows that treatment with Asc-S reduced the proliferation of ovarian cancer cells (Ov2008 and C13) by arresting them in the G2/M phase of the cell cycle. This arrest was partially due to p53 activation and to up-regulation of the p53-dependent proteins p21 and GADD45.

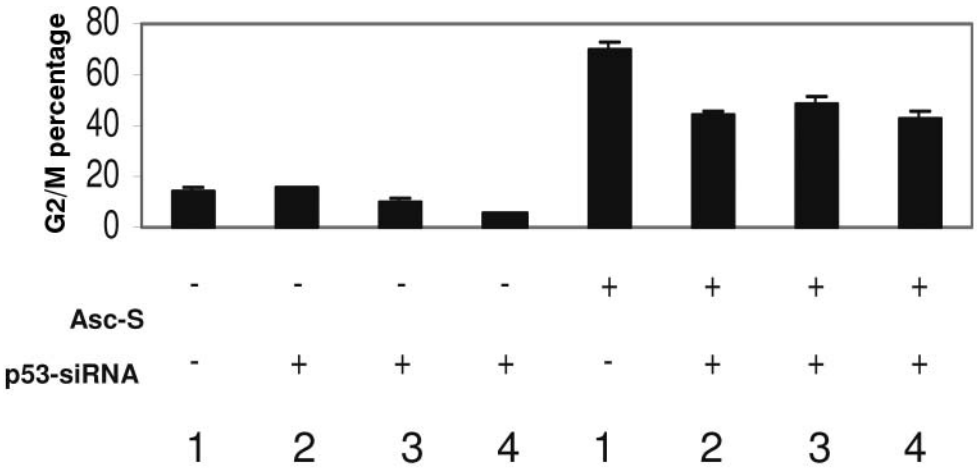

Figure 5. *Blocking of p53 in OV2008 cells (p53-si-RNA) partially rescued G2/M arrest induced by Asc-S. After treatment with 150 mM of Asc-S for 24 h, 70% of mock OV2008 cells and 40-50% of3 different p53-siRNA clones were arrested at G2/M. Lane 1: OV2008 mock; lane 2,3, and 4: p53-siRNA clones.*

Experimental, clinical and epidemiological studies have reported the role of ascorbic acid in the prevention of different types of cancer. A mixture of ascorbic acid and cupric sulfate administered orally has been shown to significantly inhibit human mammary tumor growth in mice (18). Ascorbic acid reduced the incidence of kidney tumors induced by estradiol or diethylstilbesterol in hamsters through a decrease in the formation of genotoxic metabolites (19). Ascorbic acid and its derivatives were shown to be cytotoxic and inhibitory to the growth of a number of malignant and non-malignant cells *in vitro* and *in vivo*, including neuroblastoma, osteosarcoma and retinoblastoma (20-25). A number of ascorbic acid isomers were synthesized and tested on tumor cell lines. Ascorbate-6-palmitate and ascorbate-6-stearate, the fatty acid esters of ascorbic acid were found to be more potent inhibitors of growth of murine leukemia cells compared to ascorbate-2-phosphate, ascorbate 6-phosphate or ascorbate 6-sulfate (21, 26). Naidu *et al.* have reported that ascorbyl stearate is more potent in inhibiting the growth of glioblastoma multiforme cells than water-soluble ascorbic acid (7, 11). In a previous study, we found that ascorbyl stearate had potent antiproliferative and proapoptotic effects on human pancreatic carcinoma cells, and that a decrease in proliferative activity was due to cell accumulation at the G2/M phase of the cell cycle (27).

In this study, we investigated the mechanisms involved in Asc-S-induced G2M arrest of human ovarian cancer cells. We previously showed that a set of human ovarian cancer cell lines were blocked at the G2/M phase by Asc-S (12). These lines included Ov2008, A2780 and PA-1 expressing wild-type p53. Here we observed that the human ovarian cancer cell line OV2008, and its cisplatin-resistant cell line derivate C13, expressing a wild-type form of p53, were also arrested at the G2/M phase of the cell cycle after Asc-S treatment. Therefore, because of the role of p53 in regulating G2/M cell cycle transition, we decided to use Ov2008 and C13 to define the contribution of p53 to Asc-S-induced G2/M cell cycle arrest.

It is known that p53 regulates the G2/M transition *via* modulation of the cyclin dependent kinase Cdc2, which is essential for the entry of cells into mitosis (28). Binding to cyclin B and phosphorylation at threonine 161 by CDK-activating kinase (CAK) are required to activate Cdc2 (29). During G2, the Cdc2/cyclin B complex is kept inactive by phosphorylation on tyrosine 15 and threonine 14 of Cdc2 by the kinases Wee1 and Myt1, respectively (30). At the onset of mitosis, both of these residues are dephosphorylated by the phosphatase Cdc25 (13). The Cdc2/cyclin B complex can then phosphorylate Cdc25c, further activating it and initiating a positive feedback loop. Kinases ATM and ATR, *via* activation of kinases ChK1 and ChK2, can also phosphorylate Cdc25, causing it to bind to the 14-3-3s protein. This protein sequesters Cdc25 in the cytoplasm where it cannot activate Cdc2 (31). Protein phosphatase 1 (PP1) inactivates Cdc25 by dephosphorylating the same residue that is modified by Cdc2 (32). With respect to p53, several of the transcriptional targets of p53 can inhibit Cdc2. p21 can inhibit Cdc2 by activating 14-3-3s protein, which anchors Cdc2 in the cytoplasm where it cannot induce mitosis. p53-dependent activation of GADD45 can also inhibit Cdc2 by dissociating it from cyclin B1 (14). Therefore, repression of cyclin B1 and Cdc2 by p53 enforces the arrest of the cell cycle at G2/M.

Consistent with flow cytometry data, phosphorylation of Cdc2 on tyrosine 15 was increased by Asc-S exposure, whereas no effect was observed on total Cdc2 by Western blot. To investigate the events involved in Cdc2 activation, we quantified the gene expression of *p21, GADD45, 14-3-3s,* and *Cdc25C* by Western blot. The amount of p21 and GADD45 protein increased 3- to 5-fold after exposure to Asc-S, while 14-3-3s and Cdc25c protein levels showed no changes. Moreover, phosphorylation of Cdc25c, examined immunochemically, did not change, indicating that Cdc25c is not involved in Asc-S induced G2/M arrest.

Since p21 and GADD45 are transcriptional targets of p53, we then tested the protein level of total p53 and p53 phosphorylated on serine-15. The total and phosphorylated p53 were induced by Asc-S treatment in a dose-dependent manner, supporting the hypothesis that p53 plays a significant role in Asc-S-induced G2/M arrest. Using a mobility shift assay, we further confirmed that, following the exposure to Asc-S, the induction of p53-regulated genes *p21* and *GADD45* occurs through p53. p53 was bound to consensus binding sites within the p21 promoter and the third intron of the *GADD45* gene. Blockage of p53 protein expression by siRNA resulted in reduced p21 and GADD45 expression, indicating that these genes are induced by p53 following Asc-S treatment. Our findings indicate that Asc-S has significant effects on cell cycle and induces a G2/M arrest through promotion of p53 protein synthesis and phosphorylation. However, blockage of p53 partially rescues Asc-S induced G2/M arrest, indicating that a p53 independent pathway may also be involved in inducing the Asc-S related changes.

We did not observe any elevation in phosphorylated Chk1 and Chk2, following Asc-S exposure (data not shown). This finding mitigates the hypothesis of a role of ATM/ATR in p53 activation through the Chk1 and Chk2 pathway (15) following Asc-S treatment. The past years have witnessed a growing knowledge of genetic abnormalities underlying ovarian epithelial cancer and a better appreciation in molecular terms of the clinical heterogeneity of this disease as well as a variety of therapeutic interventions. However, this cancer remains an illness with an insidious onset and a dismal prognosis. Ascorbyl stearate has clear antiproliferative activity in the absence of clinical toxicity and such properties make this derivative an attractive adjuvant compound in the treatment of human ovarian epithelial cancers, especially those expressing wild-type p53.

Acknowledgements

The authors thank Beatriz Saunders, B.S., for valuable technical assistance with cell culture studies. None of the authors had a personal or financial conflict of interest.

This work was supported by Department of Defense grant OCRP DAMD17-02-1-0670.

References

1 Jemal A, Siegel R, Ward E, Xu J, Smigal C and Thun MJ: Cancer Statistics, 2006. CA Cancer J Clic *56*: 106-130, 2006.

2 Nen RB and Cottreau C: Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst *91*: 1459-1467, 1999.

3 Liehr JG: Vitamin C reduces the incidence and severity of renal tumors induced by estradiol or diethylstilbestrol. Am J Clin Nutr *54*: 1256S-1260S, 1991.

4 Ibric LL, Peterson AR and Sevanian A: Mechanisms of ascorbic acid-induced inhibition of chemical transformation in C3H/10T1/2 cells. Am J Clin Nutr *54*: 1236S-1240S, 1991.

5 Aidoo A, Lyn-Cook LE, Lensing S and Wamer W: Ascorbic acid (vitamin C) modulates the mutagenic effects produced by an alkylating agent *in vivo*. Environ Mol Mutagen *24*: 220-228, 1994.

6 Banks WA and Kastin AJ: Peptides and blood brain barrier: lipophilicity as predictor of permeability. Brain Res Bull *15*: 287 292, 1985.

7 Naidu AK, Wiranowska M, Kori SH, Prockop LD and Kulkarni AP: Inhibition of human glioma cell proliferation and glutathione-S-transferase by ascorbyl esters and interferon. Anticancer Res *13*: 1469-1471, 1993.

8 MakinoY, Sakagami H and Takeda M: Induction of cell death by ascorbic acid derivatives in human renal carcinoma and glioblastoma cell line. Anticancer Res *19*: 3125-3132, 1999 .

9 Liu JW, Nago N, Kageyama K and Miwa N: Anti-metastatic effect of an autooxidation-resistant and lipophilic ascorbic acid derivative through inhibition of tumor invasion. Anticancer Res *20*: 113 118, 2000.

10 Naidu AK, Wiranowska M, Kori SH, Prockop LD and Kulkarni AP: Inhibition of cell proliferation and glutathione-S-transferase by ascorbyl esters and interferon in mouse glioma. J Neuro-Oncol *16*: 1-10, 1993.

11 Naidu KA, Tang JL, Naidu KA, Prockop LD, Nicosia SV and Coppola D: Antiproliferative and apoptotic effect of ascorbyl stearate in human glioblastoma multiforme cell: Modulation of insulin-like growth factor-I receptor (IGF-IR) expression. J Neuro-Oncol *54*: 15-22, 2001.

12 Fang Q, Naidu KA, Naidu KA Zhao H, Sun M, Dan HC, Nasir A, Kaiser HE, Cheng JQ, Nicosia SV and Coppola D: Ascorbyl stearate inhibits cell proliferation and tumor growth in human ovarian carcinoma cells by targeting the P12K/AKT pathway. Anticancer Res *26*: 203-209, 2006.

13 Cox LS and Lane DP: Tumor suppressors, kinases and clamps: How p53 regulates the cell cycle in response to DNA damage. BioEssays *17*: 501-508, 1995.

14 Ko LJ and Prives C: p53: puzzle and paradigm. Genes Dev *10*: 1054-1072, 1996.

15 Levine AJ: P53, the cellular gatekeeper for growth and division. Cell *88*: 323-331, 1997.

16 Denhardt DT: Signal transduction pathways and regulation of the mammalian cell cycle: cell type-dependent integration of external signals. *In*: The Molecular Basis of Cell Cycle and Growth Control. Stein G, Baserga R, Giordano A and Denhardt David T (eds.). New York, Wiley-Liss, pp. 225-304, 1999.

17 Brummelkamp TR, Bernards R and Agami R: A system for stable expression of short interfering RNAs in mammalian cells. Science *296(5567)*: 550-553, 2002.

18 Tsao CS: Inhibiting effect of ascorbic acid on growth of human mammary tumor xenografts. Am J Clin Nutr *54*: 1274S-1280S.
19 Liehr JG: Vitamin C reduces the incidence and severity of renal tumors induced by estradiol or diethylstibesterol. Am J Clin Nutr *54*: 1256S-1260S, 1991.
20 Park CH and Kimler BF: Growth modulation of human leukemic, pre-leukemic and myeloma progenitor cell by L-ascorbic acid. Am J Clin Nutr *54*: 1241S-1246S, 1991.
21 Eckert-Maksic M, Kovacek I, Maksic ZB, Osmak M and Paveli K: Effect of ascorbic acid and its derivatives on different tumors *in vivo* and *in vitro*. *In*: Molecules in Natural Science and Medicine. An Encomium for Linus Pauling. Maksic ZB and Eckert-Maksic M (eds.). New York: Ellis Horwood, pp. 509 524, 1991.
22 Murakami K, Muto N, Fukasawa GK and Yamamoto I: Comparison of ascorbic acid and ascorbic acid 2-O-L-glucosidase on the cytotoxicity and bioavailability to low density culture of fibroblast. Biochem Pharmacol *44*: 2191-2197, 1992.
23 Roomi MW, House D, Eckert-Maksic M, Maksic ZB and Tsao CS: Growth suppression of malignant leukemia cell line *in vitro* by ascorbic acid (Vitamin C) and its derivatives. Cancer Lett *122*: 93-99, 1998.
24 Pavelic K: L-ascorbic acid induced DNA strand breaks and cross links in human neuroblastoma cell. Brain Res *342*: 369-373, 1985.
25 Medina MA, de Veas RG and Schweigerer L: Ascorbic acid is cytotoxic for pediatric tumor cells cultured *in vitro*. Biochem Mol Biol Inter *34*: 871-874, 1994.
26 Roomi MW, House D and Tsao CS: Cytotoxic effect of substitution at 2-, 6-, and 2,6-positions in ascorbic acid on malignant cell line. Cancer Biochem Biophys *16*: 295-300, 1998.
27 Naidu KA, Karl RC, Naidu KA and Coppola D: Antiproliferative and proapoptotic effect of ascorbyl stearate in human pancreatic cancer cells: association with decreased expression of insulin-like growth factor 1 receptor. Dig Dis Sci *48(1)*: 230-237, 2003.
28 Nurse P: Universal control mechanism regulating onset of M-phase. Nature *344(6266)*: 503-508, 1990.
29 Pines J: Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J *308(Pt 3)*: 697-711, 1995.
30 Booher RN, Holman PS and Fattaey A: Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. J Biol Chem *272(35)*: 22300-22306, 1997.
31 Taylor WR and Stark GR: Regulation of the G2/M transition by p53. Oncogene *20(15)*: 1803-1815, 2001.
32 Walker DH, DePaoli-Roach AA and Maller JL: Multiple roles for protein phosphatase 1 in regulating the Xenopus early embryonic cell cycle. Mol Cell Biol *3*: 687-698, 1992.

Received March 12, 2007
Revised July 30, 2007
Accepted September 27, 2007