

Cancer Cell Growth and Extracellular Matrix Remodeling Mechanism of Ascorbate; Beneficial Modulation by *P. leucotomos*

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Abstract. Ascorbate has dose-dependent inverse effects on cancer cells growth and expression of matrixmetalloproteinases (MMP) and transforming growth factor- β (TGF- β), which regulate extracellular matrix (ECM) remodeling. We examined melanoma cell viability and ECM remodeling mechanisms of ascorbate and its modulation by an extract from *Polypodium leucotomos* (PL) (a fern) via the regulation of apoptosis, heat-shock proteins (HSPs), MMP-1 or tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) that inhibits MMP-1. The dose-dependent regulation of cell viability/proliferation by ascorbate was associated with inverse regulation of apoptosis and stimulation of HSPs at growth-inhibitory concentrations. PL antagonized the stimulation of MMP-1, TGF- β and HSPs by a growth-inhibitory ascorbate dose and stimulated the expression of TIMP-1, while maintaining growth inhibition. We infer that a combination of ascorbate with PL is beneficial to cancer management via the simultaneous inhibition of cell growth and expression of MMP-1, TGF- β and HSPs, and furthermore, the stimulation of TIMP-1.

The hallmarks of cancer include cell growth and metastasis, facilitated by the matrix metalloproteinases (MMPs) and transforming growth factor (TGF- β), which remodel the extracellular matrix (ECM) (1-5). The activity of MMPs is inhibited by tissue inhibitor of matrixmetalloproteinases (TIMPs).

Ascorbate (vitamin C) is a major regulator of the ECM and regulates cancer biology. It inhibits the invasiveness of several types of cancer such as gastric, oral, pulmonary, fibrosarcoma

and melanoma (6-11). It also causes the elimination of breast, oral, epidermoid, endometrial cancer cells (12-16). Conversely, tumors accumulate high concentrations of ascorbate that may give them a metabolic advantage (17, 18)

We have previously reported the dose-dependent inverse effects of ascorbate on growth versus expression of the ECM remodeling MMPs (MMP-1, MMP-2) and TGF- β (19). Ascorbate significantly inhibits cancer (renal adenocarcinoma, melanoma, mammary cancer) cell viability while stimulating MMPs and TGF- β (protein and mRNA levels) at lower concentrations, indicating elimination of cancer cells with damage to the ECM (19). Conversely, ascorbate dramatically stimulates cell proliferation and inhibits MMP and TGF- β expression at its higher concentrations, implicating growth and ECM advantage (19).

The goal of this research was to determine the growth and ECM remodeling mechanism of ascorbate via apoptosis, heat-shock proteins and TIMPs, and the counteraction of the detrimental ECM effects of the growth inhibitory ascorbate concentration by combination with an extract from *Polypodium leucotomos* (PL) (20-24). The regulation of heat-shock proteins or TIMPs by ascorbate in cancer is not reported, nor the modulation of ascorbate effects by PL. PL is rich in polyphenols and has potent skin protective effects, topically and systemically (21-24). It has antioxidant, anti-inflammatory and photoprotective properties: inhibiting oxidative stress, lipid peroxidation, dermal mast cell infiltration, inflammatory cytokines, DNA damage and UV induced tumors (21-24). In addition, PL inhibits MMP-1 expression in fibroblasts and keratinocytes (22).

Specifically, the research examined the dose-dependent regulation of the apoptosis-regulating proteins (p53, p21, Bax, caspase-3) by ascorbate; the effects of growth inhibitory ascorbate concentrations on expression of TIMP-1 and heat-shock proteins (HSP-27, 70, 90), which stabilize proteins and facilitate tumorigenesis; and the counteraction of the detrimental effects of a growth inhibitory ascorbate dose (expression of MMP-1, TIMP-1, TGF- β and HSPs) by PL.

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Key Words: Vitamin C, cancer, viability, matrix metalloproteinases, tissue inhibitor of matrix metalloproteinases, transforming growth factor-beta, *Polypodium leucotomos*.

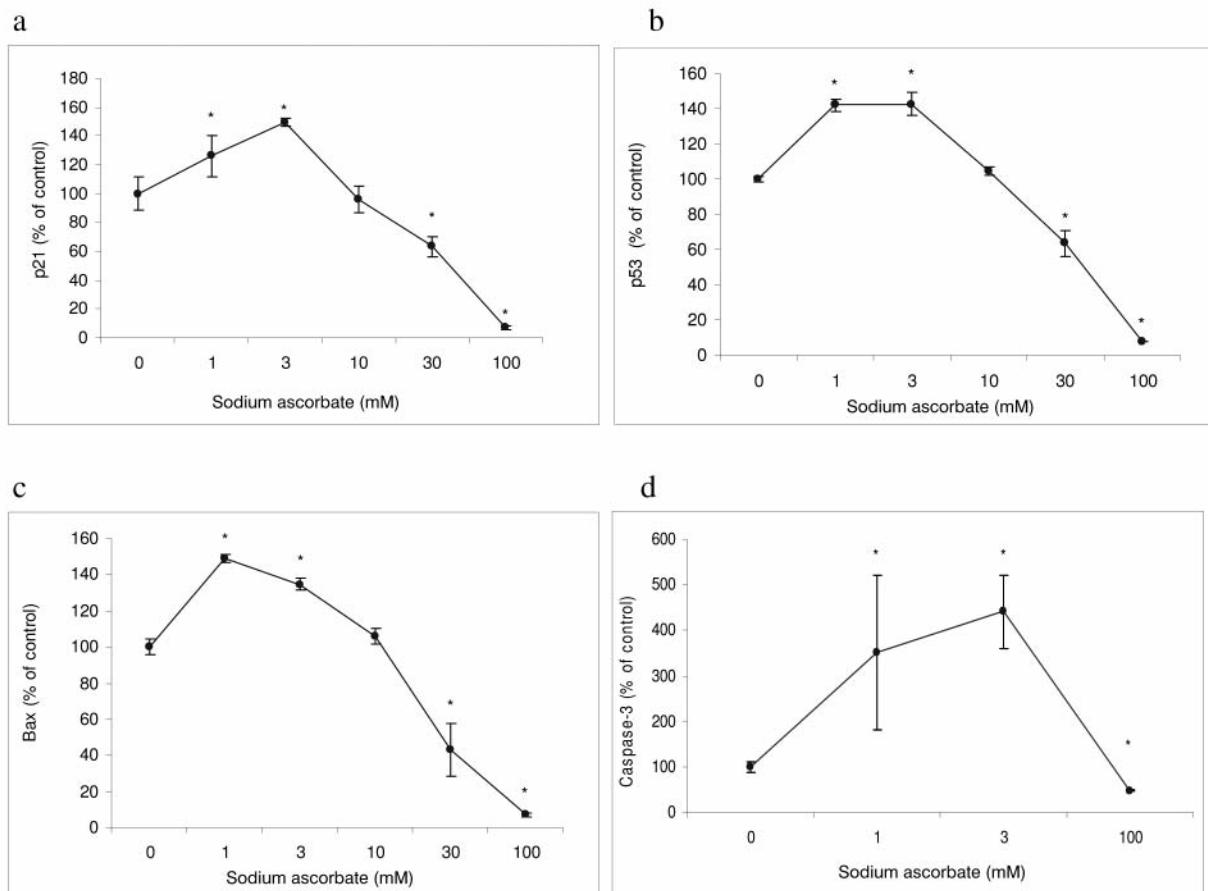


Figure 1. *Inverse dose-dependent effects of ascorbate on apoptosis.* Melanoma cells were dosed with ascorbate at 0, 1, 3, 10, 30 or 100 mM for 24 hours, and examined for expression of p53 (a), p21 (b), Bax (c), and caspase-3 activity (d). * $p<0.05$, relative to control. Error bars represent standard deviation, $n=4$.

Materials and Methods

Cell culture. Human melanoma cell lines were obtained from American Tissue Cell Culture and propagated in Dulbecco's modified Eagle's minimal media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. For experiments, cells dosed with 0-100 mM ascorbate with or without 0.5% PL or specific small interfering RNA (negative control siRNA or MMP-1 siRNAs, Invitrogen) in DMEM supplemented with 1% serum replacement (Sigma). The cells were examined for cell viability, apoptosis-inducing proteins (p53, p21, Bax, caspase-3 activity), and heat-shock proteins (HSP-27, 70, 90). The media were examined for MMP-1, TIMP-1 and TGF- β protein levels.

Cell viability. The cell viability was examined by CellTiter 96® Aqueous One or MTS assay (Promega) by incubating cells with aliquots of MTS mixture (yellow) for 30 minutes at 37°C. Viable cells reduce the MTS mixture to produce a color change (brown), read spectrophotometrically at 490 nm.

ELISA. The protein levels were determined by ELISA (apoptosis proteins: Sigma; heat-shock proteins: StressGen; TIMP-1 and MMP-1: Chemicon and Kirkgaard and Perry Laboratories, Inc; TGF- β : R&D Systems). 100 μ L aliquots of test samples were added to a target-specific 96-well plates for 24 hours at 4°C. The wells were blocked with bovine serum albumin, and then incubated with respective antibodies for 1 hour at room temperature. The plate was washed with wash buffer, incubated with secondary antibody linked to peroxidase for 1 hour at room temperature, washed and subsequently incubated with peroxidase substrate until color development, measured spectrophotometrically at 405 nm.

CPP32 (Caspase-3) protease activity. Aliquots of cell lysates (50 μ L) were incubated with an equal volume (50 μ L) of the reaction buffer and 5 μ L of 7-amino-4-trifluoromethyl coumarin (AFC-DEVD) for 1 hour at 37°C (Clontech). The shift in fluorescence emission of AFC-DEVD, on its proteolysis to free AFC by the protease, was detected in a fluorometer, using a 400 nm excitation filter and 505 nm emission filter.

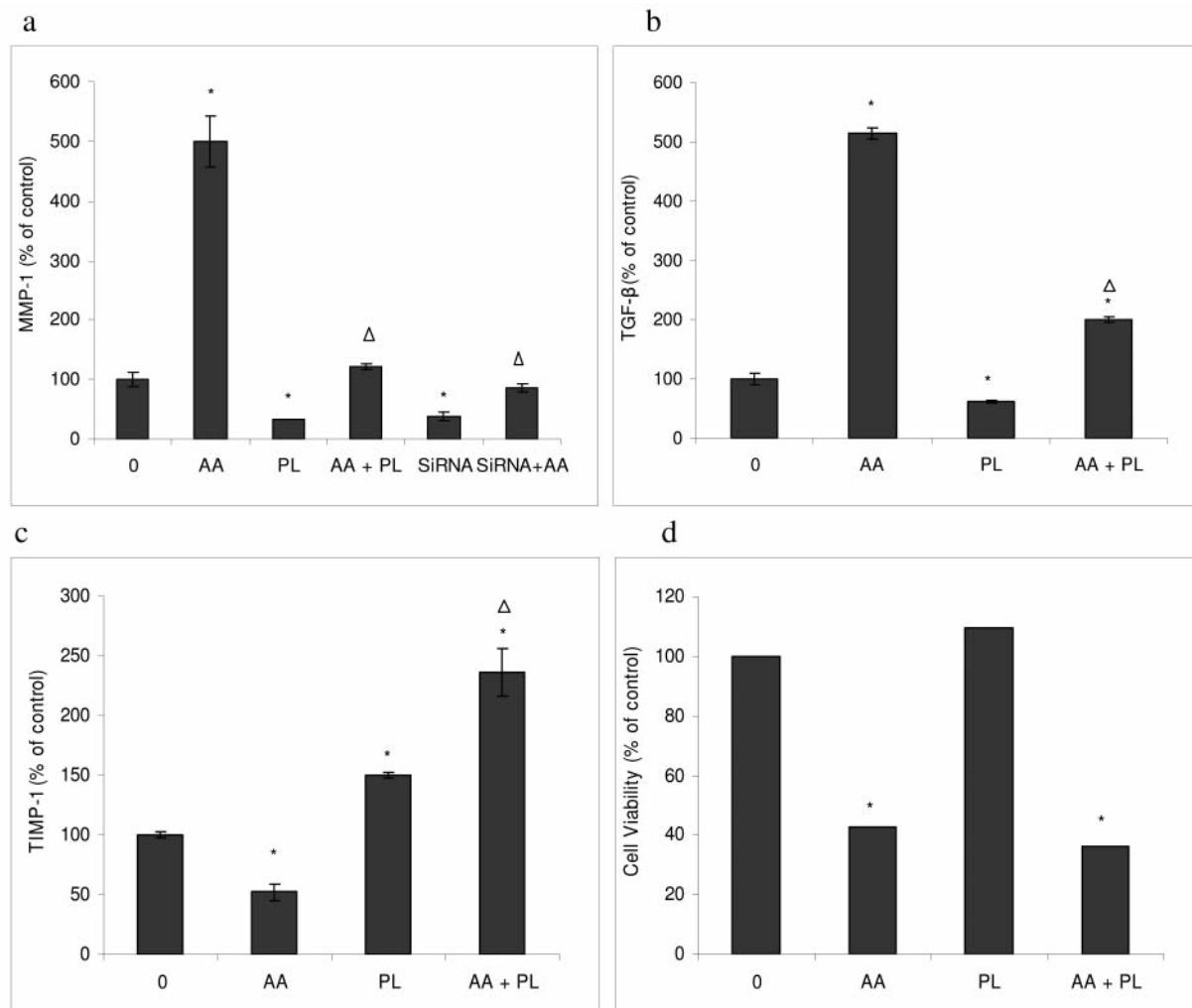


Figure 2. Antagonistic effects of a growth-inhibitory ascorbate concentration and *P. leucotomos* extract on MMP-1, TIMP-1 and TGF- β expression but not cell viability. Cells were incubated with 0 or 3 mM ascorbate (AA), 0.5% *P. leucotomos* extract (PL) or a combination of 3 mM AA and 0.5% PL (AA+PL) for 24 hours, and the expression of MMP-1 (a), TIMP-1 (b), TGF- β (c), and cell viability (d) were assessed. Cells were transfected with MMP-1 siRNA for 24 hours, dosed with 0 or 3 mM ascorbate and subsequently examined for MMP-1 expression (a). * $p<0.05$, from control (0); Δ $p<0.05$, from AA effect for AA+PL. Error bars represent standard deviation, $n=4$.

RNA interference. Melanoma cells were transfected with 0 or 100 nM specific stealth siRNA, following preliminary dose response assays with 0–300 nM siRNA, using lipofectamine (Invitrogen), dosed with or without 3 mM ascorbate for 24 hours and examined for MMP-1 expression (25). The transfected siRNAs were negative control stealth siRNA (12935200; Invitrogen, data not shown) or MMP-1 stealth siRNAs (HSS 12935200; HSS 106611; Invitrogen).

Statistical analysis. The data are represented as a percentage of the control (represented at 100%) with the control being cells not treated with ascorbate. The data were statistically analyzed by ANOVA and Student's *t*-tests at the 95% confidence interval for significant differences from control and from ascorbate (AA) for combination (AA + PL) effect.

Results

Ascorbate exhibits dose-dependent differential regulation of cell viability/proliferation (19). These dose-dependent effects on cell viability/proliferation are inversely related to the expression or activity of apoptosis-modulating proteins (Figure 1). Ascorbate at its growth-inhibitory concentrations (1 and 3 mM) significantly stimulated the expression of p53, p21 and Bax up to 150% of control, and caspase-3 activity up to 440% of control ($p<0.05$) (Figure 1a-d). Conversely, ascorbate at its growth-stimulatory concentrations of 30 and 100 mM, respectively, inhibited the expression of p53, p21

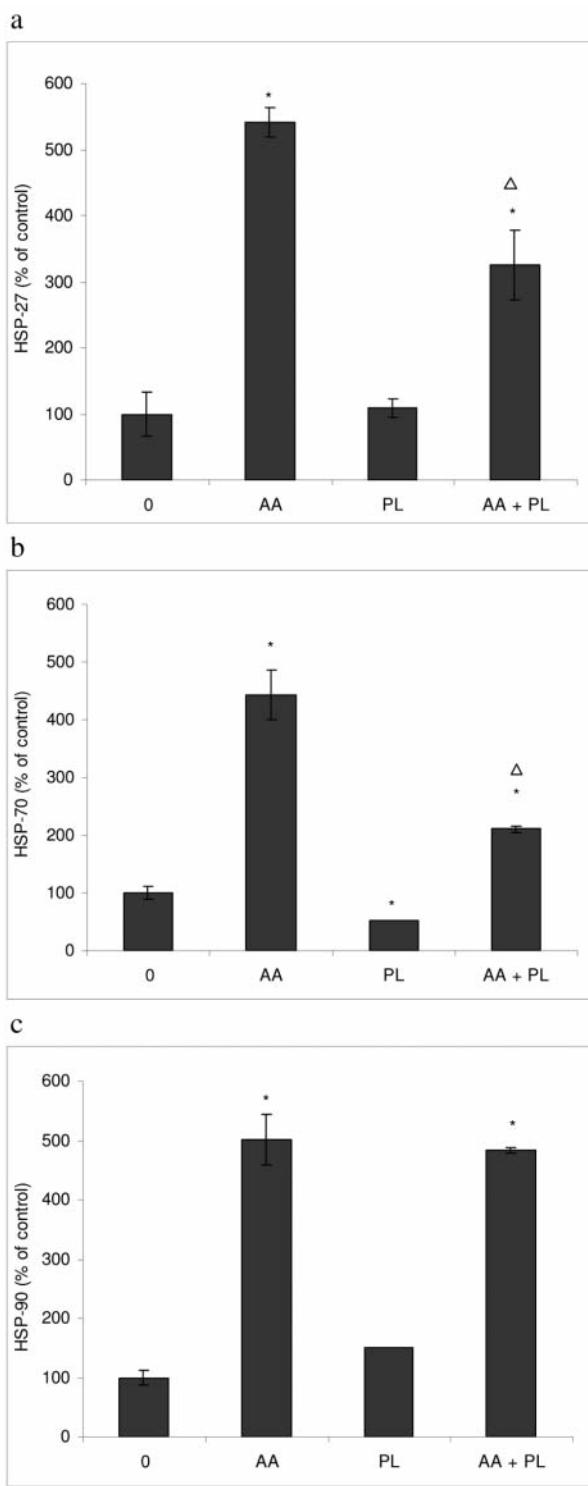


Figure 3. Combination effects of a growth-inhibitory ascorbate concentration and *P. leucotomos* extract on heat-shock proteins. Cells were incubated with 0 or 3 mM ascorbate (AA), 0.5% *P. leucotomos* extract (PL) or a combination of 3 mM AA and 0.5% PL (AA+PL) for 24 hours, and examined for the expression of HSP-27 (a), HSP-70 (b), and HSP-90 (c). * $p<0.05$, from control (0); $\triangle p<0.05$, from AA effect for AA+PL. Error bars represent standard deviation, n=4.

and Bax to up to 40%, and about 7% of control ($p<0.05$) (Figure 1a-c) (21). Caspase-3 activity was inhibited by 30 mM ascorbate to 47% of control ($p<0.05$) (Figure 1d).

PL counteracted the stimulation of MMP-1 expression by a growth-inhibitory ascorbate concentration (3 mM) to control levels, as did *MMP-1* siRNA, and lowered its stimulation of TGF- β expression by 60% ($p<0.05$, relative to ascorbate effect) (Figure 2a, b). Furthermore, while the growth inhibitory ascorbate dose (3 mM) inhibited TIMP-1 expression to 50% of control, in combination with PL, TIMP-1 was stimulated to 236% of control (450% of ascorbate effect) ($p<0.05$, relative to control, and ascorbate effect) (Figure 2c). PL maintained the growth inhibitory effect of ascorbate (Figure 2d).

Ascorbate at the growth-inhibitory concentrations significantly stimulated the expression of heat-shock proteins (HSP-27, 70 and 90) up to 541% of control, respectively ($p<0.05$) (Figure 3). PL significantly inhibited the ascorbate stimulation of HSP-27 and HSP-70 up to 50% of the ascorbate effect but did not alter the regulation of HSP-90 ($p<0.05$, relative to ascorbate effect) by ascorbate (Figure 3).

Discussion

We have herein extended our research on the reciprocal effects of ascorbate on cell growth and the expression of MMP and TGF- β (19).

Ascorbate regulates cell growth by apoptotic and non-apoptotic mechanisms (26, 27). The growth-inhibitory ascorbate concentrations induced expression or activity of p53, p21, Bax and caspase-3, whereas the growth-stimulatory doses of ascorbate inhibited the expression or activity of these proteins. An increase in the expression of p53 and its downstream protein p21 activates pro-apoptotic Bax protein that triggers apoptosis (20). We did not examine cell cycle arrest, which may be another mechanism for the growth inhibition or survival of the viable cells.

The growth-inhibitory ascorbate doses also increased expression of HSP-27, 70 and 90, indicating a stress response, oxidative or cell-differentiation mechanism in growth inhibition by acorbate. The HSPs are induced on cellular stress and have similar and specific effects. While HSP-70 is constitutively expressed, HSP-27 induces differentiation and HSP-90 stabilizes oncogenic proteins, including MMPs (28-33). It may be inferred that cell viability, despite the induction of apoptosis-inducing proteins, and ECM remodeling potential via the increased expression of MMPs and TGF- β , in the viable ascorbate dosed cells is from the induction of HSPs. PL lowered the induction of HSP-27 and HSP-70 by the growth-inhibitory ascorbate concentration to about 50% but did not alter regulation of HSP-90 by ascorbate. The regulation of HSP-70 by PL may be the result of its immunomodulatory effects (23, 24).

The beneficial effect of ascorbate on growth inhibition is offset by its stimulation of MMPs and inhibition of TIMP-1, suggesting increased metastatic potential to the surviving ascorbate-dosed melanoma cells. However, the MMP-1 stimulation by the growth-inhibitory ascorbate dose is counteracted by PL along with stimulation of TIMP-1, without alteration of growth inhibition, suggesting combination supplementation in cancer therapy. PL was as effective as *MMP-1* siRNA in counteracting the induction of MMP-1 in melanoma cells. MMPs are critical targets in the inhibition of cancer metastasis (35-40).

The induction of TGF- β is another integral factor to tumor pathology via induction of MMPs, angiogenesis and cancer metastasis. The inhibition of TGF- β expression is a target for cancer therapy (41). PL significantly lowered TGF- β stimulation by a growth-inhibitory ascorbate dose, further supporting combination supplementation as beneficial in cancer management.

In summary, the dose-dependent differential regulation of cell viability/proliferation and by ascorbate is associated with inverse regulation of apoptosis and heat-shock proteins. The detrimental effects of the growth inhibitory ascorbate concentration on ECM remodeling are counteracted by PL via inhibition of MMP-1 and TGF- β and stimulation of TIMP-1, suggesting combination supplementation as being beneficial in cancer therapy.

Acknowledgements

We thank Sagar Chauhan, Hyeondo Hwang and Marvin Tuason for graphing / the assistance with formatting literature cited.

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*Received February 9, 2009**Accepted May 5, 2009*