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In Vivo Antitumor Effect of Ascorbic Acid, Lysine, Proline and Green Tea Extract on Human Prostate Cancer PC-3 Xenografts in Nude Mice: Evaluation of Tumor Growth and Immunohistochemistry

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Abstract. *Background: Matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), Ki 67 (proliferative protein) and constituents of ECM play a critical role in angiogenesis, and are crucial in neoplastic invasion and metastasis. Based on the antitumor properties of certain nutrients, we investigated the effect of a diet containing lysine, proline, arginine, ascorbic acid and green tea extract on the growth of tumors induced by implanting human prostate cancer PC-3 cells in athymic nude mice and on the expression of MMPs, VEGF, Ki 67 and fibronectin in these tumors, as well as the production of mucin (by PAS staining). Materials and Methods: Male nude mice (n=12) were inoculated with $3x10^6$ prostate cancer PC-3 cells and randomly divided into two groups; Group A was fed a regular diet and Group B was fed a regular diet supplemented with 0.5% of the nutrient mixture (NM). Four weeks later, tumors were excised, weighed and processed for histology. Results: The results showed inhibition of tumor growth in Group B. Histological studies revealed inhibition of MMP-9 and VEGF secretion and mitosis in Group B tissues. Conclusion: Nutrient supplementation strongly suppressed the growth of tumors without any adverse effects in nude mice, suggesting strong potential as an anticancer agent.*

Prostate cancer, the number one incident cancer in men and the second most deadly cancer in the United States, primarily affects males age 55 and older and is more common in African American males than Caucasian males (1). While surgery is the treatment of choice for early stage prostate cancer, it is limited to small, localized tumors and is associated with a number of side-effects. Hormonal therapy, which seeks to stop or slow the progression of prostate cancer either by blocking the action of cancer-supporting male hormones or stopping their production, is only effective for a few years before the cancer is able to spread independently of male hormones, and is associated with side-effects such as impotence, hot flashes, nausea, vomiting, diarrhea, liver complications, weakened bones, breast growth and/or tenderness (2,3). Local radiation therapy is fairly effective at eradicating early stage prostate cancer but, because there are often delays in the start of radiotherapy from the date of diagnosis, cancer cell growth in the interim often eliminates the use of this treatment in Stage I prostate cancer (4). Finally, external radiotherapy focuses on cancer cell destruction, but does not address metastases (5,6). It not only has been ineffective in providing a cure, but also indiscriminately attacks all cells, causing cellular damage and destruction of the body's connective tissue, and thus facilitates cancer metastasis. Clearly, current treatment modalities are limited in both the treatment of prostate cancer and prevention of metastasis, creating a profound need for new safe and effective therapeutic approaches.

Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through various matrix metalloproteinases (MMPs). Studies, conducted *in vivo* and *in vitro* on metastasized prostate cancer to bone, revealed that MMPs play a significant role in both metastatic tumor growth and bone matrix turnover (7). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. In 1992, Rath and Pauling postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to modulate tumor growth and expansion (8). These nutrients can exercise their antitumor effect through the inhibition of MMPs and strengthening of connective tissue surrounding cancer cells (tumor encapsulating effect).

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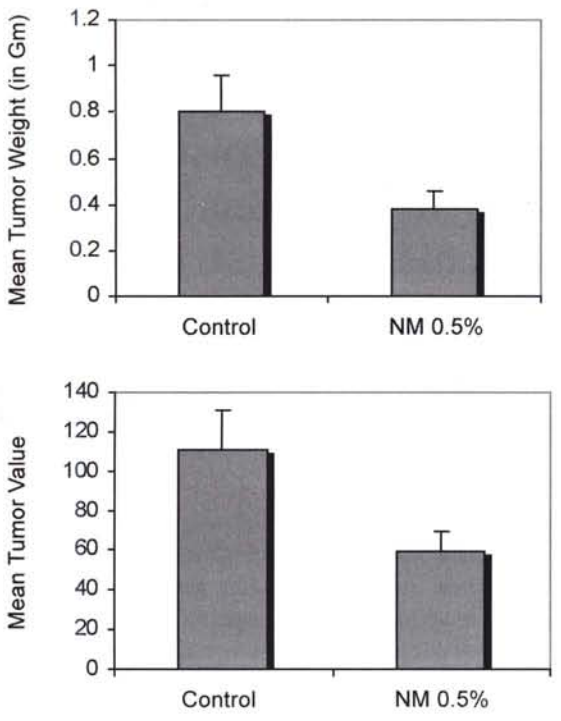

Figure 1. *Effect of supplementation with nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract on prostate cancer tumor xenografts in male nude mice (A). Effect of supplementation with nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract on mean tumor value (in mm^2) of prostate cancer cell xenografts (B).*

In this study, we investigated the antitumor potential of a mixture containing ascorbic acid, lysine, proline and green tea extract on human prostate PC-3 cells *in vivo* (xenograft in male nude mice).

Materials and Methods

Cancer cell lines and culture. Human prostate cancer PC-3 cells, obtained from ATCC (American Type Culture Collection, Rockville, MD, USA), were maintained in Ham's F12 medium (F12K), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY, USA. At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a density of $3x10^6$ cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA, USA) for inoculation.

Animals. Male athymic nude mice (NCr-nu/nu), approximately six weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA, USA, and maintained in microinsulator cages under pathogen-free conditions on a 12-hour light/12-hour dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of

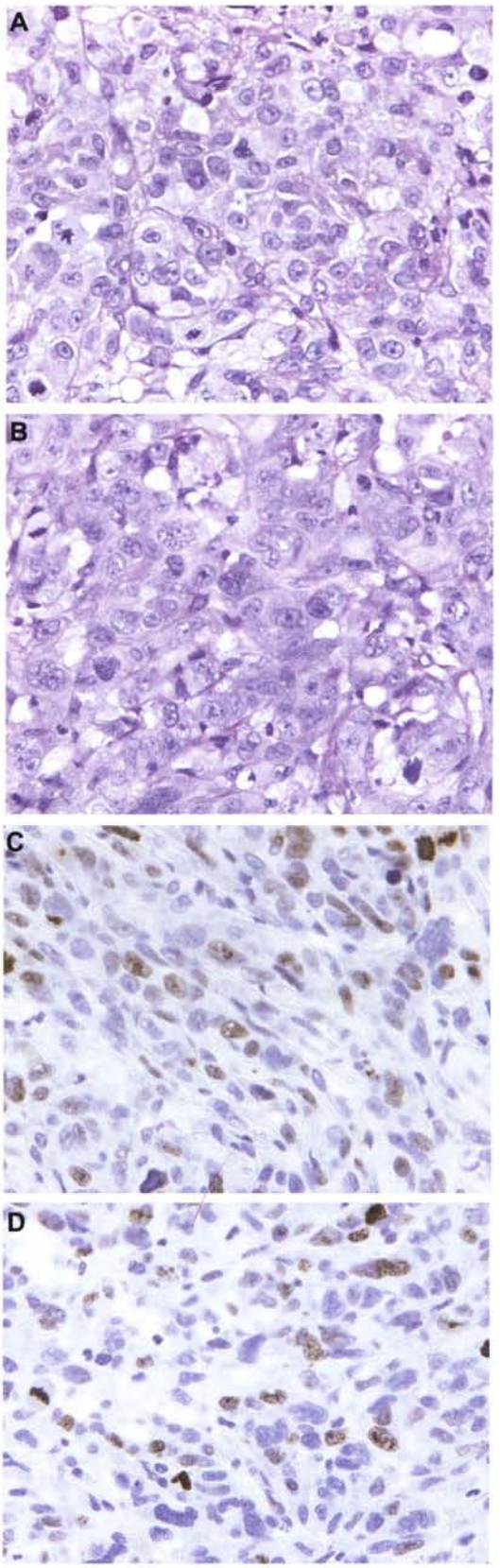

Figure 2.

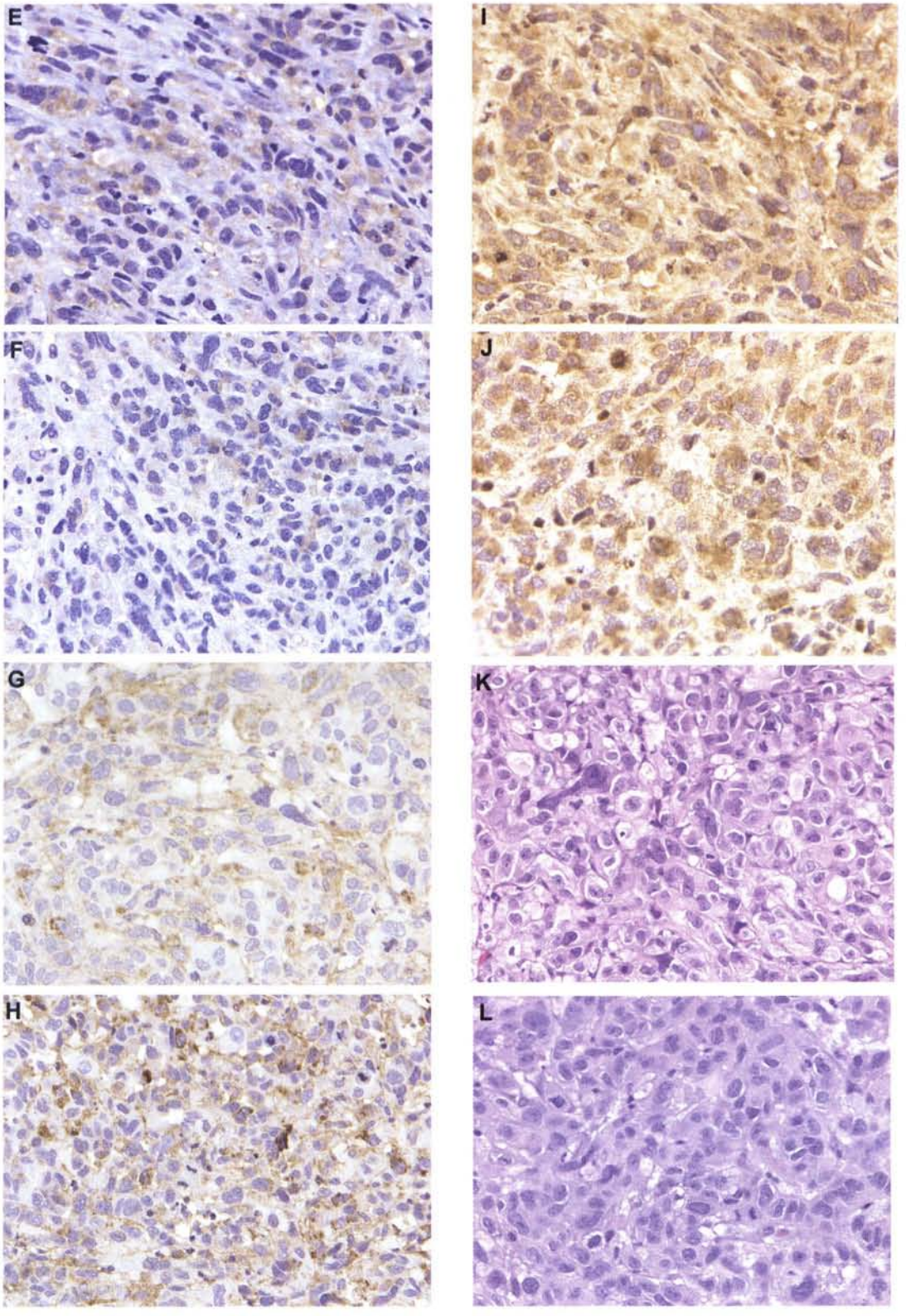

Figure 2. *Effect of nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract on prostate PC-3 xenograft tissue in nude mice: cytochemistry and immunohistochemistry of control and nutrient-supplemented tumor tissue (A - PAS Control, B – PAS NM 0.5%, C - Ki 67 Control, D – Ki 67 NM 0.5%, E – VEGF Control, F – VEGF NM 0.5%, G – Fibronectin Control, H – Fibronectin NM 0.5%, I – MMP-9 Control, J – MMP-9 NM 0.5%, K – H&E Control, L – H&E NM 0.5%).*

experimental animals. After housing for a week, the mice were inoculated with $3x10^6$ human prostate cancer cells in 0.2 ml of PBS and 0.1 ml of Matrigel. After injection, the mice were randomly divided into two groups, A and B. Six mice were allocated to each group. From day one, mice from Group A were fed a regular diet, while those in Group B were fed a regular diet supplemented with 0.5% nutrient mixture (NM). After four weeks, the mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin and processed for histology. The dimensions [length (L) x width (W)] of the tumors was measured with a pair of digital calipers, and the tumor value was calculated using the following formula: ½ x L x W.

Cytochemistry and immunohistochemistry. Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4-5 microns. The sections were deparaffinized through xylene and graduated alcohol series to water, and incubated for 5 minutes in aqueous 3% hydrogen peroxide to block endogenous peroxidase. Histological sections were stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) stains for evaluation using a standard light microscope.

Immunochemical studies were performed on formalin-fixed, paraffin-embedded sections. Standard immunohistochemical staining procedures were used for staining antibodies. After deparaffinization and appropriate epitope retrieval, the sections were incubated with primary antibody. Detection was by biotinylated goat anti-mouse antibodies followed by streptavidin conjugated to horseradish peroxidase with the use of diaminobenzidine as the chromogen. The polyclonal rabbit anti-human antibodies used for MMP-9, VEGF, fibronectin and Ki 67 were obtained from Santa Cruz Biotechnology, Inc., CA, USA, and from Sigma.

Composition of the nutrient mixture (NM). The stock solution of the nutrient mixture (total weight 4.4 g) used for testing was composed of the following: vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1 mg.

Statistical analysis. The results were expressed as means±SD for the groups. The data were analyzed by independent sample "*t*" test.

Results

Tumor growth. The results showed that the nutrient-supplemented nude mice developed significantly smaller tumors (reduction in weight by 47% $p<0.0001$) and less vascular ones than did the control group of nude mice (Figure 1A). Nude mice from both groups showed no weight loss over the study period. Treatment with the nutrient formulation resulted in a significantly decreased mean tumor value in nude mice with human colon cancer cell xenografts studied over the 4-week treatment period, at 53% ($p=0.0002$ - Figure 1B).

Cytochemistry and immunohistochemistry. PAS staining, a measure of mucin, showed increased PAS material in the tumor tissue of the control group of mice (Figures 2A-B). The Ki 67 level, a measure of cell proliferation, was greater for the control group (Figures 2C-D). There is a strong positive correlation between high Ki 67 index and high-grade histopathology in neoplasms. VEGF staining, an indicator of angiogenesis, was higher in the control than in the supplemented group (Figures 2E-F). Fibronectin material was higher in the supplemented group (Figures 2G-H). Fibronectin exists in two main forms: as an insoluble glycoprotein dimer that serves as a linker in the ECM and as a soluble disulphide linked dimer found in the plasma. Fibronectin sometimes serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. The control tissue cytoplasmic staining for MMP-9 was greater than in the supplemented mouse tissue (Figures 2I-J). There was no difference between control and supplemented tissue on H&E staining (Figures 2K-L).

Discussion

The results of this study demonstrated a significant suppression of prostate tumor growth in immune-impaired (athymic) male nude mice by supplementation with 0.5% of the nutrient mixture (composed of ascorbic acid, lysine, proline and green tea extract). Histological examination demonstrated a reduction in mitotic index and MMP-9 and VEGF material, as well as decreased PAS (mucin) material in the tissue of supplemented animals. Furthermore, our previous *in vitro* study demonstrated substantial inhibition of human prostate PC-3 cancer cell Matrigel invasion and migration (reduced by 80% at 500 μg/ml and totally inhibited at 1000 μg/ml NM ($p=0.0001$)), and dose-dependent inhibition of MMP-2 and MMP-9 expressions by the nutrient combination (PC-3 by zymography showed two bands corresponding to MMP-2 and MMP-9 activity; NM produced virtually total inhibition at 100 μg/ml and 500 μg/ml concentrations, respectively) clearly demonstrating its antimetastatic activity (9).

Degradation of basement membranes by MMPs is the key to the invasive potential of cancer cells. Research has shown that highly metastatic cancer cells secrete higher amounts of MMPs than do poorly metastatic cells, demonstrating that the invasive and metastatic abilities of these cancer cells *in vitro* and *in vivo* correlates with MMP-9 expression (10). MMP activity can also be affected by lysine through plasmin-mediated mechanisms. Lysine can interfere with the activation of plasminogen into plasmin by plasminogen activator by binding to plasminogen active sites (8). Matrix invasion can also be controlled by increased connective tissue strength and stability, contributing to the "encapsulation" of the tumor, secondary to the synergistic activity of the nutrients. Optimization of the synthesis and structure of collagen fibrils depends upon hydroxylation of proline and lysine residues in collagen fibers. It is well known

that ascorbic acid is essential for the hydroxylation of these amino acids, as well as for collagen synthesis. Lysine is the most abundant amino acid in collagen. Neither ascorbic acid nor lysine are produced in the human body, therefore sub-optimal levels of these nutrients is possible in various pathological stages and through deficient diets.

The inhibitory effects of the individual nutrients composing the nutrient mixture have been reported in both clinical and experimental studies. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines (11-13); in addition, low levels of ascorbic acid have been reported in cancer patients (14-16). Green tea extract is a potent anticancer agent that has been reported to have a growth inhibitory effect against human cancer cell lines (17-20). However, individual nutrients are not as powerful as nutrient synergy. Our previous studies demonstrated that the synergistic anticancer effect of ascorbic acid, proline, lysine and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients (21).

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both prostate cancer prevention and treatment, the results of these studies suggest that the formulation of green tea extract, lysine, proline and ascorbic acid is an excellent candidate for adjunctive therapeutic use in the treatment of highly metastatic prostate cancer, by inhibiting MMP expression and invasion without toxic effects.

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