

***In vivo* antitumor effect of ascorbic acid, lysine, proline and green tea extract on human colon cancer cell HCT 116 xenografts in nude mice: Evaluation of tumor growth and immunohistochemistry**

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Abstract. Colorectal cancer is the second most deadly cancer in the United States. When diagnosed early, current treatments bring a limited success; however, once metastasis occurs, radiation and chemotherapy are generally ineffective. Structural changes in the ECM are necessary for cell migration during tissue remodeling. Matrix metalloproteinases (MMPs), VEGF, Ki-67 (proliferative protein), and constituents of ECM, such as fibronectin, play a critical role in angiogenesis and are thus crucial in neoplastic invasion and metastasis. Based on antitumor properties of certain nutrients, we investigated the effect of a diet containing lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on the growth of tumors, induced by implanting human colon HCT 116 cancer cells in athymic nude mice, and the expression of MMPs, VEGF, Ki-67 and fibronectin in these tumors, as well as the production of mucin (by PAS staining). After one week of isolation, 5 to 6 week-old athymic male nude mice (n=12) were inoculated with 3×10^6 colon cancer HCT 116 cells. After injection, the mice were randomly divided into 2 groups; group A was fed a regular diet and group B was fed a regular diet supplemented with 0.5% NM. The mice were sacrificed 4 weeks later, and their tumors were excised, weighed, and processed for histology. Results showed that the nutrient mixture (NM) inhibited growth and reduced the size of tumors in nude mice. Furthermore, histological evaluation revealed increased mitotic index, MMP-9 and VEGF secretion and reduced basement membrane in the control group tissues. Nutrient supplementation strongly suppressed the growth of tumors without any adverse effects in nude mice, suggesting the nutrient combination has potential as an anticancer agent. Histological studies supported these findings by showing

inhibition of MMP-9 and VEGF secretion and mitotic index, which are critical parameters for cancer control and prevention.

Introduction

Colorectal cancer, the second most deadly cancer in the United States, will claim the lives of approximately 56,000 Americans this year. Colon cancer affects both men and women over the age of 50 with approximately the same frequency. While colorectal cancer is very treatable upon early detection, 5-year survival is <10% once the cancer metastasizes to the lymph, liver or other areas, and most of these fatalities are associated with metastasis (1).

Early stage colon cancer is, generally, successfully treated with surgery (local excision/colon resection) depending on the size of the lesion. However, side effects can range from mild to severe: diarrhea, constipation, depression, bleeding, infection, and 15% of all colorectal patients require a permanent colostomy (2). Standard treatment of Stage II colon cancer and advanced stages consist of both chemotherapy and radiation therapy. As with most chemotherapy approaches, cancer cells are eventually capable of independent growth, invasion, adhesion, angiogenesis and avoidance of apoptosis, rendering this approach ineffectual (3). Only 10-20% patients on fluorouracil experience palliation, yet the associated side effects of chemotherapy include nausea, vomiting, hair loss, mouth sores, diarrhea, fatigue, bleeding, infection, and weight loss (2). Finally, radiotherapy may be used before surgery to shrink tumors or after surgery to eradicate any remaining cancer cells. External radiotherapy focuses on cancer cell destruction, but not metastases, which is the main cause of death in patients with colorectal cancer (4). 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, facilitating cancer metastasis. Clearly, there is a need for safe and effective therapeutic approaches that can be used to control the process of cancer metastasis as well as prevention of colon cancer.

Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and

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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 (5). These nutrients can exercise their antitumor effect through the inhibition of MMPs and strengthening connective tissue surrounding cancer cells (tumor encapsulating effect). In a previous study, we demonstrated the anti-proliferative and anti-invasive potential of lysine, ascorbic acid, proline and green tea extract on human colon cell cancer HCT 116 (6).

In this study, we investigated the antitumor potential of a mixture containing ascorbic acid, lysine, proline, and green tea extract (NM) on human colon cancer cells HCT 116 *in vivo* (xenograft in male nude mice).

Materials and methods

Cancer cell lines and culture. Human colon cancer HCT 116 cells obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in MEM culture, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY. At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a concentration of 3×10^6 cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA) for inoculation.

Animals. Male athymic nude mice (NCR-nu/nu), approximately six weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA and maintained in microinsulator cages under pathogen-free conditions on a 12-h light/dark schedule for one week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for a week, the mice were inoculated with 3×10^6 human colon cancer cells in 0.2 ml of PBS and 0.1 ml of Matrigel. After injection, the mice were randomly divided into 2 groups, A and B. There were 6 mice allocated to each group. From day 1, mice from group A were fed a regular diet, and those in group B were fed a regular diet supplemented with 0.5% NM. After 4 weeks, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin and processed for histology. 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: $1/2 \times L \times W$.

Cytochemistry and immunohistochemistry. Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4-5 microns. Sections were deparaffinized through xylene and graduated alcohol series to water, and incubated for 5 min 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.

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded sections. We used standard

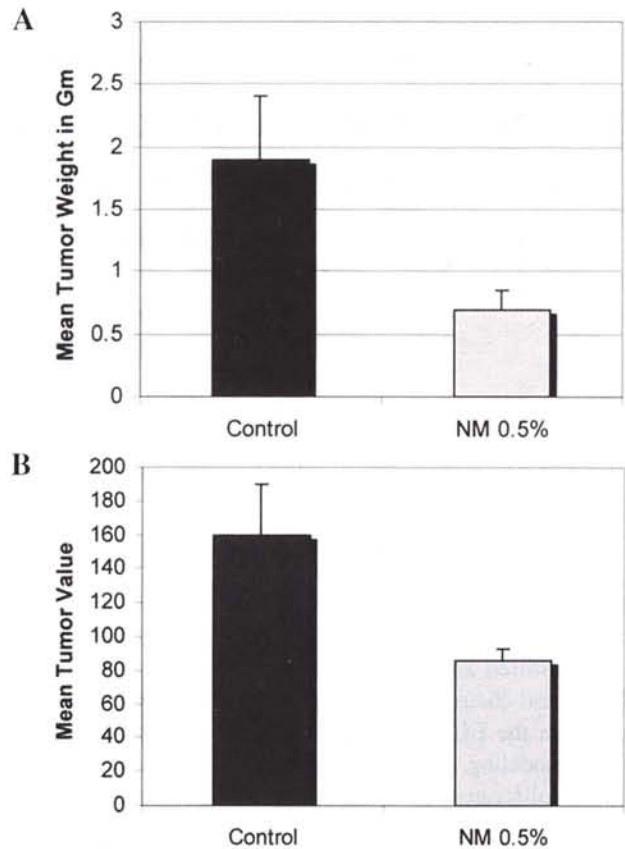


Figure 1. (A) Effect of supplementation with 0.5% NM on mean tumor weight of colon cancer HCT xenografts in nude mice. (B) Effect of supplementation with 0.5% NM on mean tumor value of colon cancer HCT116 xenografts in nude mice.

immunohistochemical staining procedures 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. Polyclonal rabbit anti-human antibodies used for MMP-9, MMP-2, VEGF, fibronectin and Ki-67 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma.

Composition of the nutrient mixture (NM). Stock solution of the nutrient mixture (total weight 4.4 Gm) 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, and manganese 1 mg.

Statistical analysis. The results were expressed as means \pm SD for the groups. Data were analyzed by independent sample t-test.

Results

Tumor growth. Results showed that the nutrient-supplemented nude mice developed significantly smaller (by 63%; $p=0.0002$),

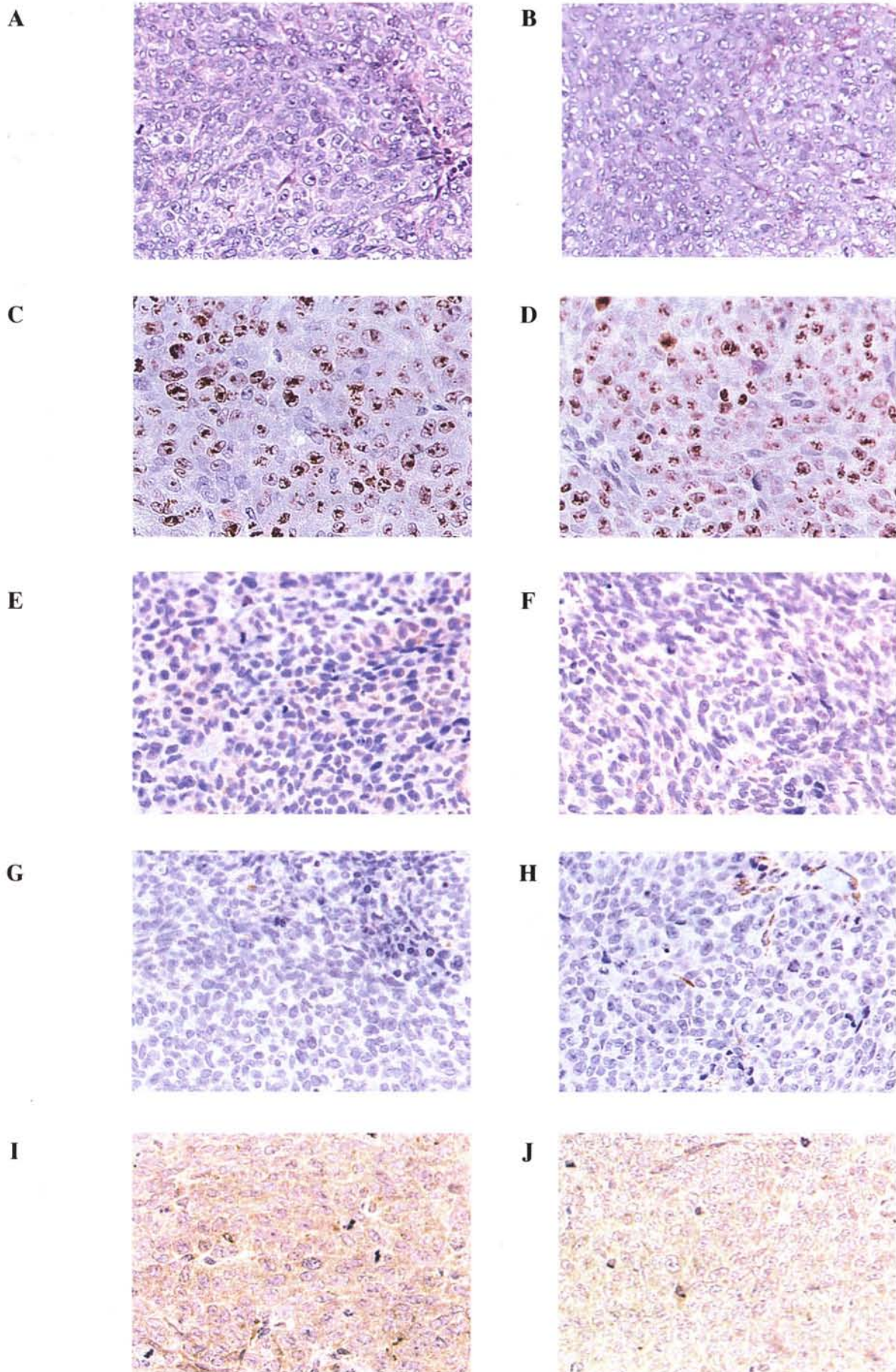


Figure 2. Effect of nutrient supplementation on colon cancer HCT 116 xenograft tissue: cytochemistry and immunohistochemistry of control and supplemented tumor tissue. (A) PAS, control; (B) PAS, NM 0.5%; (C) Ki-67, control; (D) Ki-67, NM 0.5%; (E) VEGF, control; (F) VEGF, NM 0.5%; (G) fibronectin, control; (H) fibronectin, NM 0.5%; (I) MMP-9, control; and (J) MMP-9, NM 0.5%.

and less vascular tumors than those in the control group of nude mice (Fig. 1A). Nude mice from both groups showed no weight loss over the study period. Treatment with the nutrient formulation resulted in significantly decreased mean tumor value in nude mice with human colon cancer cell xenografts studied over the 4-week treatment period by 46% ($p=0.0005$). See Fig. 1B.

Cytochemistry and immunohistochemistry. PAS staining, a measure of mucin, showed increased PAS material in the tumor tissue of the control group of mice (Fig. 2A and B). Ki-67 level was similar for both groups (70-80%). See Fig. 2C and D. There is a strong positive correlation between the high Ki-67 index and high-grade histopathology of neoplasms. VEGF staining, an indicator of angiogenesis, was higher in the control than in the supplemented group (Fig. 2E and F). Fibronectin material was higher in the supplemented group (Fig. 2G and H). Fibronectin exists in two main forms: an insoluble glycoprotein dimer that serves as a linker in the ECM, and 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. Control tissue cytoplasmic staining for MMP-9 was greater than in the supplemented mouse tissue (Fig. 2I and J).

Discussion

The results of this study demonstrated significant suppression of colon tumor growth in immune-impaired (athymic) male nude mice by supplementation with 0.5% of the nutrient mixture (which contains ascorbic acid, lysine, proline, and green tea extract). Histological examination demonstrated reduction in MMP-9 and VEGF material, as well as increased fibronectin (glycoprotein) and decreased PAS (mucin) material in the tissue of supplemented animals. Furthermore, our previous *in vitro* study demonstrated substantial inhibition (76% at 100 $\mu\text{g/ml}$ concentration of NM) of human colon cancer cell HCT 116 invasion and MMP-9 expression by this nutrient combination, clearly demonstrating its antimetastatic activity (7).

Degradation of basement membranes by MMPs is key to the invasive potential of cancer cells. Moreover, research has shown that highly metastatic colon cells (LuM1) secrete higher amounts of MMP-9 than do poorly metastatic cells, demonstrating that the level of tumoral invasion correlates with MMP-9 expression in colon cancer (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 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. Both ascorbic acid and lysine are not 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 (9-11); in addition, low levels of ascorbic acid have been reported in cancer patients (12-14). Green tea extract is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines (15-18). 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 (19).

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

Acknowledgements

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