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Original Article

Effect of Ascorbic Acid, Lysine, Proline, and Green Tea Extract on Human Osteosarcoma Cell Line MNNG-HOS Xenografts in Nude Mice

Evaluation of Tumor Growth and Immunohistochemistry

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Abstract

Structural changes in the extracellular matrix (ECM) are necessary for cell migration during tissue remodeling. MMPs, VEGF, Ki-67 (proliferative protein), and constituents of ECM play a critical role in angiogenesis and underlie neoplastic invasion and metastasis. This prompted us to investigate 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 osteosarcoma MNNG 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). We also investigated the effect of the supplemented diet on serum ascorbic acid, total protein content, alkaline phosphatase activity, and liver enzymes. Athymic male nude mice (n = 12) were inoculated with 3×10^6 osteosarcoma cells MNNG-HOS and randomly divided into group A (fed a regular diet) and group B (fed a regular diet supplemented with 0.5% NM). Four weeks later, the mice were sacrificed. Results showed that NM inhibited the growth and reduced the size of tumors in nude mice. Histological evaluation revealed increased mitotic index, MMP-9, and VEGF secretion in the control group tissues. Results demonstrate that the nutrient mixture of lysine, proline, arginine, ascorbic acid, and green tea extract tested strongly suppressed the growth of tumors without adverse effects in nude mice, suggesting potential as an anticancer agent.

Key Words: Osteosarcoma; MNNG-HOS; green tea extract; ascorbic acid; xenograft; MMP-9.

Introduction

Osteosarcoma, a primary malignant tumor of bone or soft parts that arises from bone-forming mesenchymal cells, primarily develops in the distal femur, the proximal tibia, the proximal humerus, and the distal radius. Classic osteosarcoma demonstrates aggressive, rapid growth with a high risk of local, "skip" metastases and early, pulmonary metastasis. It is the most common bone cancer and the sixth most common cancer in children, and is more frequent in males than females. Most osteosarcomas arise from non-inherited errors in the DNA of growing bone cells. Because these errors occur randomly and unpredictably, there is currently no effective way to prevent this type of cancer *(1)*.

For decades, standard treatment for osteosarcoma has consisted of surgery (amputation or limb salvage

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surgery) and chemotherapy, which focus on cancer cell destruction, but do not address metastasis. Radiation and chemotherapy have not only been ineffective in providing a cure, but also indiscriminately attack all cells, causing cellular damage and destruction of the body's connective tissue, and thus facilitate cancer metastasis. For example, of 31 patients studied with localized osteosarcoma *(2)* and treated with conventional chemotherapy (high-dose methotrexate and leucovarin rescue in 3 patients and intraarterial cisplatin in 28 patients) at the Anderson Cancer Center, only 3 patients did not experience local recurrence or pulmonary metastases during the follow-up period of 225+ mo. Side effects of chemotherapy include anemia, abnormal bleeding, increased risk of infection due to destruction of bone marrow, liver and kidney damage, heart problems, and hearing loss. Approximately 20% of children diagnosed with osteosarcoma have an advanced stage of osteosarcoma that has metastasized to the lungs, brain, and other bones *(1)*. Even resection of the primary tumor has been reported to potentiate distant metastasis in osteosarcoma *(3)*. Clearly, there is a need for safe and effective therapeutic approaches to control the process of cancer metastasis.

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 invasiveness of the cancer. Rath and Pauling *(4)* 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. These nutrients can exercise their antitumor effect through the inhibition of MMPs, and, in addition, by strengthening of connective tissue surrounding cancer cells through their effect on collagen synthesis. These two processes are essential for a tumor-encapsulating effect.

Our previous studies have demonstrated significant antitumoral activity of lysine, proline, ascorbic acid, and green tea extract (NM) against a number of cancer cell lines *(5)*. Additionally, this nutrient mixture has shown a strong inhibitory effect on cancer cell proliferation, their tissue invasion, and MMP secretion in vitro *(6–8)* and in vivo *(9–11)*. In this study, we investigated the antitumor potential of a unique formulation containing ascorbic acid, lysine, proline, arginine, and green tea extract on human osteosarcoma cell line MNNG-HOS in vivo (xenograft in male nude mice).

Materials and Methods

Cancer Cell Lines and Culture

Human osteosarcoma cells MNNG-HOS 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), approx 6 wk 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/12-h dark schedule for 1 wk. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for 1 wk, the mice were inoculated with 3×10^6 human osteosarcoma MNNG-HOS 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 d 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 wk, the mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin, and processed for histology.

Serum Chemistry

At termination, the mice were anesthetized and blood was collected by cardiac puncture. Blood samples were centrifuged at 4°C to separate serum and stored at –80°C. Serum ascorbic acid concentration was determined in our laboratory using HPLC. Other serum analyses (alkaline phosphatase, total protein,

albumin, globulins, SGPT, and SGOT) were performed at IDEX, Sacramento, CA, a commercial diagnostic laboratory.

Immuohistochemistry

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4-5 µm. 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.

Immunochemical studies were performed on formalin-fixed, paraffin-embedded sections. We used standard 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., CA, and Sigma.

Composition of the Nutrient Mixture (NM)

Stock solution of the nutrient mixture (total weight 4.2 g) is 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 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Lab. The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, and caffeine 1.0%); selenium 30 mg; copper 2 mg; manganese 1 mg.

The nutrient mixture (NM) was formulated based on targeting different stages of cancer progression and metastasis. For example, the ECM integrity is dependent on adequate collagen formation; the amino acids lysine and proline are necessary for formation of collagen chains and ascorbic acid is essential for the hydroxylation reaction. Manganese and copper are also essential for collagen formation. Ascorbic acid has also been shown to inhibit cell division and growth through production of hydrogen peroxide *(12)*. Green tea extract has been shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression *(13)*. *N*-acetyl cysteine has been observed to inhibit MMP-9 activity *(14)* and invasive activities of tumor cells *(15)*. Selenium has been shown to interfere with MMP secretion and tumor invasion *(16)*, as well as migration of endothelial cells through ECM *(15)*. Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells *(17)*.

Based on the evidence available in the literature and our own research, we hypothesized that a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetyl cysteine, selenium, copper, and manganese would work synergistically. For example, we found that a combination of ascorbic acid, lysine, and proline used with EGCG enhanced the anti-invasive activity of 20 µg/mL EGCG to that of 50 µg/mL *(18)*. Thus by including nutrients like *N*-acetyl cysteine, arginine, selenium, manganese, and copper in addition to ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG.

Statistical Analysis

The results were expressed as means ± 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 tumors (by 53%, $p = 0.0001$) and less vascular ones than did the control group of nude mice (Fig. 1). Food consumption and daily weight gained did not significantly differ between the groups.

Cytochemistry and Immunochemistry

PAS staining, a measure of mucin, showed increased PAS material in the tumor tissue of the control group of mice (Figs. 2A, B). Ki-6 7 (nuclear

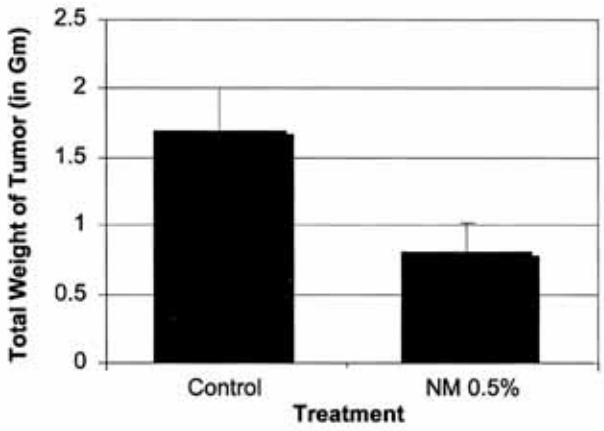


Fig. 1. Effect of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on total weight of osteosarcoma MNNG xenografts in male nude mice.

antigen preferentially expressed in proliferating cells throughout the cell cycle: G1, S, G2 and M phases, but not in resting:G_0) was higher in the control (60–70%) in contrast to the supplemented mice (40–50%). See Figs. 2C, 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 (Figs. 2E, F). No significant difference was found in fibronectin material (1–2+ in intact areas in both groups) (Figs. 2G, H). Fibronectin exists in two main forms: as insoluble glycoprotein dimer that serves as a linker in the ECM and as a soluble disulfide 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 (Figs. 2I, J).

Serum Chemistry of Animals

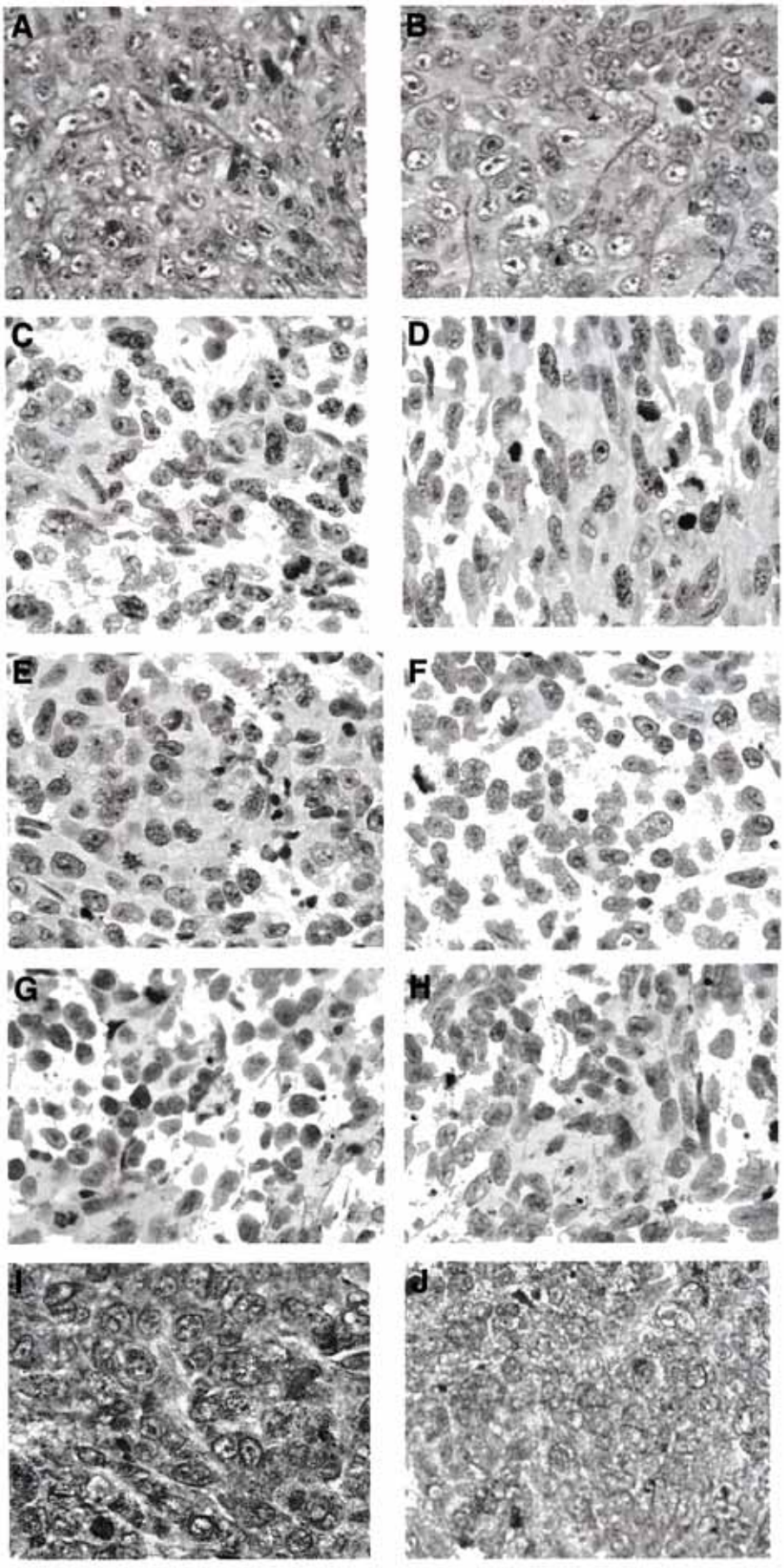
Variation in ascorbic acid intake was reflected in the serum ascorbic acid content and was significantly different in the control and test groups (Fig. 3A). Serum alkaline phosphatase levels were significantly ($p = 0.0001$) elevated in nude mice supplemented with the nutrient mixture (NM) compared to the control group. Total protein level was slightly lower with NM treatment (control: 5.6±0.16 g/dL; supplemented 5.3±0.07 g/dL), $p = 0.002$); albumin was slightly higher (control 2.23±0.17, supplemented 2.64±0.05; $p = 0.0002$), and globulin lower (control 3.4±0.16, supplemented 2.64±0.11; $p < 0.0001$) with supplementation (Fig. 3B). The mean AST and ALT values did not significantly differ in the two groups (ALT: 50 IU/L for both control and NM-treated mice; AST:482±73 for control and 395±32 for supplemented; $p = 0.02$).

Discussion

The results of this study demonstrated significant suppression of osteosarcoma 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 inhibition of MMP-9, VEGF, and mitotic index with supplementation with the nutrient mixture, all factors crucial to neoplastic cell proliferation and invasion. Serum chemistry confirmed significantly increased ascorbic acid levels and alkaline phosphatase levels with nutrient treatment.

Matrix invasion can be controlled by inhibition of MMP secretion as well as 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 on 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, suboptimal levels of these nutrients are

Fig. 2. Effect of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on tumor tissues from nude mice with osteosarcoma MNNG xenografts (Figure 2A – PAS – control, Figure 2B – PAS– NM 0.5%, Figure 2C – Ki-67-Control, Figure 2D – Ki-67- NM 0.5%, Figure 2E – VEGF – Control, Figure 2F - VEGF - NM 0.5%, Figure 2G – Fibronectin – Control, Figure 2H – Fibronectin –NM 0.5%, Figure 2I - MMP-9 – Control, Figure 2J - MMP-9 – NM0.5%).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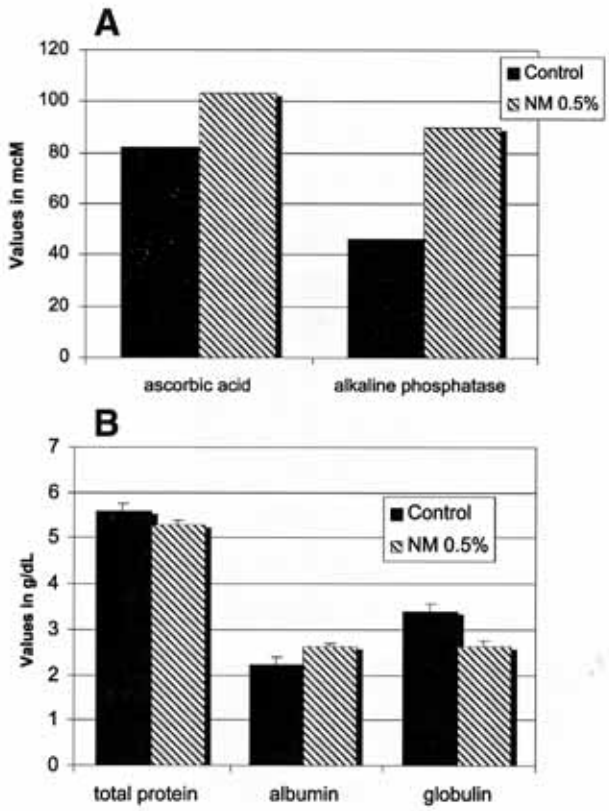

Fig. 3. Effect of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on serum ascorbic acid and alkaline phosphatase in male nude mice with MNNG/HOS subcutaneous xenografts (3A). Effect of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (NM) on serum total protein, albumin and globulin in male nude mice with MNNG/HOS subcutaneous xenograft (3B).

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 *(19–21);* in addition, low levels of ascorbic acid have been reported in cancer patients *(22–24).* ECGC is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines *(25–27).* 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 *(5).*

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of this study suggest the nutrient mixture of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate tested, as an excellent candidate for therapeutic use in the treatment of the highly aggressive osteosarcoma cancer, by suppressing tumor growth independent of immune system function and inhibiting critical steps in cancer metastasis, such as MMP secretion and invasion.

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