IN VIVO AND IN VITRO ANTITUMOR EFFECT OF NUTRIENT SYNERGY ON HUMAN OSTEOSARCOMA CELL LINE MNNG-HOS

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1 The nutrient mixture (NS) used in this study, Epicran Forté™, is a proprietary formulation provided by Matthias Rath Inc.

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Abstract:
Current treatment of osteosarcoma is associated with poor prognosis, especially due to the increased risk of developing other cancers with chemotherapy. Therefore, new safe effective treatment strategies are needed. This prompted us to investigate the synergistic effect of a nutrient mixture (NS) of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on the growth of human osteosarcoma xenografts in athymic nude mice. We also investigated the effect of NS on human osteosarcoma cell line MNNG-HOS in vitro by measuring: cytotoxicity, modulation of MMP-2 and -9, cancer cell invasive potential, and angiogenesis. After one-week of isolation, 5–6 week old athymic male nude mice (n=12) were inoculated with 3x10⁴ osteosarcoma cells MNNG-HOS. After injection, the mice were randomly divided into two subgroups; group A was fed a regular diet and group B was fed a regular diet supplemented with 0.5% of the nutrient mixture. Four weeks later, the mice were sacrificed, and their tumors were excised, weighed, and processed for histology. Cell proliferation was evaluated by MTT assay, MMP expression by gelatinase zymography, and invasion through Matrigel. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) to study enhanced MMP expression. Results showed that the nutrient mixture (NS) inhibited the growth and
reduced the size of tumors in nude mice. Furthermore, the mitotic index was decreased in the supplemented group (4-5) in contrast to the control group (12-15). The invasion of osteosarcoma MNNG-HOS cells through Matrigel was significantly reduced in a dose-dependent fashion, with 100% inhibition of invasion of MNNG cells at 50 μg/ml concentration of the nutrient mixture. No significant anti-proliferative effect by NS was seen with MNNG cells. Zymography showed dose-dependent inhibition of MMP secretion by MNNG in the presence of NS. Nutrient synergy strongly suppressed the growth of tumors without any adverse effects in nude mice, suggesting the nutrient combination has potential as an anticancer agent. In vitro studies demonstrated inhibition of cancer cell invasion and secretion of MMPs - critical parameters for cancer control and prevention.

**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 [Miller, Dowshen et al. (2002)].

For decades, standard treatment for osteosarcoma has consisted of surgery (amputation or limb salvage 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 [Jaffe, Carrasco et al. (2002)] and treated with conventional chemotherapy (high-dose methotrexate and leucovorin rescue in 3 patients and intra-arterial 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+ months. 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 [Miller, Dowshen et al. (2002)]. Even resection of the primary tumor has been reported to potentiate distant
metastasis in osteosarcoma [Tsunemi, Nagoya et al. (2003)]. 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 [Rath and Pauling (1992)] 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 anti-tumor 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.

In this study, we investigated the anti-tumor potential of a unique formulation containing ascorbic acid, lysine, proline, arginine and EGCG on human osteosarcoma cell line MNNG-HOS in vivo (xenograft in male nude mice) and in vitro (by measuring: cell proliferation, modulation of matrix metalloproteinases (MMPs), and invasive potential.

Materials and Methods:

In Vivo Study
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 3x10^6 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 microisolator 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 experimental animals. After housing for a week, the mice were inoculated with 3x10^6 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 day one, mice from Group A were fed a regular diet and those in Group B were fed a regular diet supplemented with 0.5% NS. After four weeks, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin and processed for histology.

In Vitro Study

Cancer Cell Line and Culture

Human osteosarcoma cells were grown in MEM in 24-well tissue culture plates (Costar, Cambridge, MA). Cell cultures were supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated with 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO₂. At near confluence, the cells were treated with the nutrient mixture (NS) dissolved in media and tested at 0, 10, 100, 500, and 1000 µg/ml in triplicate at each dose. A group of cells were also treated with PMA 200 ng/ml. The plates were then returned to the incubator. Cell proliferation was evaluated 24 hrs following incubation with test reagents.

MTT Assay

Cell proliferation was evaluated based on the MTT assay. The MTT assay [Masman JT, (1983)] is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. After MTT addition (0.5mg/ml) the plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD₅₇₀ of the DMSO solution in each well was considered to be proportional to the number of cells. The OD₅₇₀ of the control (treatment without supplement) was considered 100%.

Gelatinase Zymography

MMP expression in condition media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel (Invitrogen Corporation) in the presence of 0.1% gelatin. Culture media (20µl) was loaded and SDS-PAGE was performed with a tris-glycine SDS buffer. After electrophoresis, the gels were washed with 5% Triton X-100 for 30 minutes. The gels were then incubated for 24 hours at 37°C in the presence of 50mM Tris-HCl, 5mM CaCl₂, 5µM ZnCl₂, PH 7.5 and stained with Coomassie Blue R 0.5% for 30 minutes and
destained. Protein standards were run concurrently and approximate molecular weights were determined.

**Matrigel Invasion Studies**

Invasion studies were conducted using Matrigel™ (Becton Dickinson) inserts in 24-well plates. Suspended in medium, osteosarcoma cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO₂ for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with Hematoxylin and Eosin and visually counted under the microscope.

**Composition of the Nutrient Mixture (NS)** *

Stock solution of the nutrient mixture (total weight 4.4 Gm) 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 (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1mg.

**Statistical Analysis**

The results were expressed as means ± SD for the groups. Data was analyzed by independent sample “t” test.

**Results:**

**In Vivo Study**

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 (Figure 1A). Furthermore, histological examination revealed that mitotic figures in the control group averaged 12-15 per high power field; in contrast, the nutrient supplemented rats developed tumors with a decreased mitotic figure (4-5 per high-power field) (Figure 1B). The tumors, located in the subcutaneous layer, were expansile, with evidence of peripheral invasion. The neoplasm was composed of spindle shaped or irregularly round cells with large, irregularly round to oval hyperchromatic nuclei and scant cytoplasm with indistinct borders. Irregular areas of tumor necrosis involved about 70% of the tumor mass.
Figure 1A – Effect of NS on total weight of osteosarcoma MNNG xenografts in male nude mice

Figure 1B – Histology of tumor tissue in supplemented (Suppl) and control mice

Control 10x Mitotic figures  Control 40x Mitotic figures  Control 10x Angiogenesis

Suppl 10x Mitotic figures  Suppl 40x Mitotic figures

A = angiogenesis
MF = mitotic figure
Figure 1C - Photographs of sample tumors from control and supplemented nude mice

Control Group #1  Control Group #2  Supplemented #1  Supplemented #2

In Vitro Study

Osteosarcoma Proliferation Study

The nutrient mixture (NS) showed no significant antiproliferative effect on MNNG-HOS (Figure 2) untreated or PMA-induced cells ($p=0.2$).

![Graph showing cell growth of MNNG-HOS proliferation](image)

Figure 2- Effect of the nutrient mixture (NS) and PMA on human osteosarcoma cells MNNG-HOS proliferation

Gelatinase Zymography Study

As shown in Figure 2, zymography demonstrated only expression of MMP-2 by human osteosarcoma cells MNNG-HOS cell line, with total virtual inhibition at 500 µg/ml.
Figure 2(A) – Effect of exposure to nutrient mixture (NS) on MMP-2 expression by human osteosarcoma MNNG HOS cells

Unstimulated MNNG HOS cells  PMA (200 ng/ml)-Treated MNNG HOS cells

1-Markers, 2- Control, 3-7 NS 10, 50, 100, 500, 1000 µg/ml

Invasion Study
Invasion of MNNG-HOS osteosarcoma cells through Matrigel was dramatically reduced at 10 µg/ml (87%), 97% at 50 µg/ml and totally inhibited at 500 µg/ml (p=0.0002).

Figure 3 – Effect of the nutrient mixture (NS) on Matrigel invasion and migration by human osteosarcoma cells MNNG-HOS

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
epigallocatechin gallate). Furthermore nutrient supplementation resulted in decreased mitotic index when contrasted with the control mice. These results are consistent with the results of the in vitro studies that demonstrated significant inhibition of MMP-2 secretion and matrix invasion by osteosarcoma MNNG cells by the synergistic effect of the nutrients in this mixture.

Matrix invasion can be controlled by inhibition of MMP expression 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 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 antimitastatic actions on malignant cell lines [Koh, Lee et al. (1998), Roomi, House et al. (1998), Naidu, Karl et al.(2003)]; in addition, low levels of ascorbic acid have been reported in cancer patients [Anthony and Schorah (1982), Nunez, Ortiz de Apodaca et al. (1995), Kurbacher, Wagner et al. (1996)]. ECGC is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines [Valcic, Timmerman et. al. (1996), Mukhtar and Ahmed (2000), Yang, Liao et al. (1998)]. 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 ECGC on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients [Netke, Roomi et al.(2003)].

Our results suggest that nutrient synergy is an excellent candidate for therapeutic use in the treatment of the highly aggressive osteosarcoma cancer, by suppressing tumor growth independent of immune system function, inhibiting critical steps in cancer metastasis, such as MMP expression and invasion.

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References


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