Antitumor effect of nutrient synergy on human osteosarcoma cells U-2OS, MNNG-HOS and Ewing's sarcoma SK-ES.1

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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 and effective treatment strategies are needed. We investigated the effect of a unique mixture of nutrients containing lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (EGCG) on human osteosarcoma cell lines U-2OS, MNNG-HOS, and Ewing's sarcoma SK-ES-1 by measuring: cell proliferation, expression of matrix metalloproteinase-2 (MMP-2), MMP-9, and invasive and angiogenesis potential. Cell proliferation was evaluated by MTT assay, matrix metalloproteinases (MMP) expression by gelatinase zymography, VEGF expression by ELISA, and invasion through Matrigel. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) to study enhanced MMP and VEGF expression. The invasion of osteosarcoma U-2OS and MNNG-HOS cells through Matrigel was significantly reduced in a dose-dependent fashion, with 100% inhibition of invasion of U-2OS cells at 100 µg/ml, and MNNG cells at 50 µg/ml concentration of the synergistically acting nutrient mixture. Ewing's sarcoma SK-ES-1 cells were not invasive. Nutrient synergy (NS) exhibited a dose response antiproliferative effect on osteosarcoma U-2OS cells, reaching 67% at 1000 µg/ml of NS; no significant suppression of cell proliferation was seen with MNNG or Ewing's sarcoma cells. Zymography showed dose-dependent inhibition of MMP secretion by all three cell lines in the presence of NS. VEGF secretion by U-2OS cells was completely blocked at 500 µg/ml of NS. Our results suggest NS is an excellent candidate for therapeutic use in the treatment of osteosarcoma, by inhibiting cancer cell invasion, and secretion of MMPs and VEGF, all critical parameters for cancer control and prevention.

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Introduction

Osteosarcoma, a primary malignant tumor of bone or soft parts that arises from bone-forming mesenchymal cells, primarily develops in the distal femur, proximal tibia, proximal humerus and 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, sixth most common cancer in children, and 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). Another bone cancer, Ewing's sarcoma, develops in immature nerve tissue in bone marrow and also occurs more frequently in children and adolescents.

For decades, standard treatment of 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 facilitating cancer metastasis. For example, of 31 patients studied with localized osteosarcoma (2) 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, menstrual irregularities, bladder inflammation and bleeding into the urine, skin and 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 that can be used 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 anti-tumor effect through the inhibition of MMPs and, in addition, their effect on collagen synthesis by strengthening connective tissue surrounding cancer cells. These two processes are essential for a tumor encapsulating effect.

In a previous study, we demonstrated the antiproliferative and anti-invasive potential of lysine, ascorbic acid, proline and epigallocatechin gallate (EGCG) on human breast cancer (MDA-MB 231), colon cell cancer (HCT 116) and melanoma (A2058) cell lines (5). Nutrient synergy (NS) also suppressed the growth of these tumors, without any adverse effects, in nude mice. In the current study, we investigated the anti-tumor potential of the nutrient mixture *in vitro* on several human osteosarcoma cell lines: U-2OS, MNNG-HOS, and Ewing's sarcoma SK-ES-1, by measuring cytotoxicity, modulation of matrix metalloproteinases (MMPs), MMP-2 and MMP-9, and invasive potential and angiogenesis by measuring VEGF secretion.

Materials and methods

Cell culture. Human osteosarcoma cells U-2 OS, MNNG-HOS, and Ewing's sarcoma SK-ES-1 were obtained from ATCC (American Type Culture Collection, Rockville, MD). U-2OS and Ewing's sarcoma were grown in McCoy medium and MNNG-HOS 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 µg/ml). Cells were incubated with 1 ml of media at 37˚C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with the nutrient mixture 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. The cytotoxicity was evaluated after 24 h following incubation with test reagents.

MTT assay. Nutrient effects on osteosarcoma cell proliferation were evaluated by MTT assay. The MTT assay (6) is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. After MTT addition (0.5 mg/ml) the plates were covered and returned to the 37˚C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in I ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} 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 (20 µl) 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 min. The gels were then incubated for 24 h at 37˚C in the presence of 50 mM Tris-HCl, 5 mM $CaCl_2$, 5 µM $ZnCl_2$, pH 7.5 and stained with Coomassie Blue R 0.5% for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined.

Matrigel invasion studies. Invasion studies were conducted using Matrigel™ inserts (Becton-Dickinson) 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.

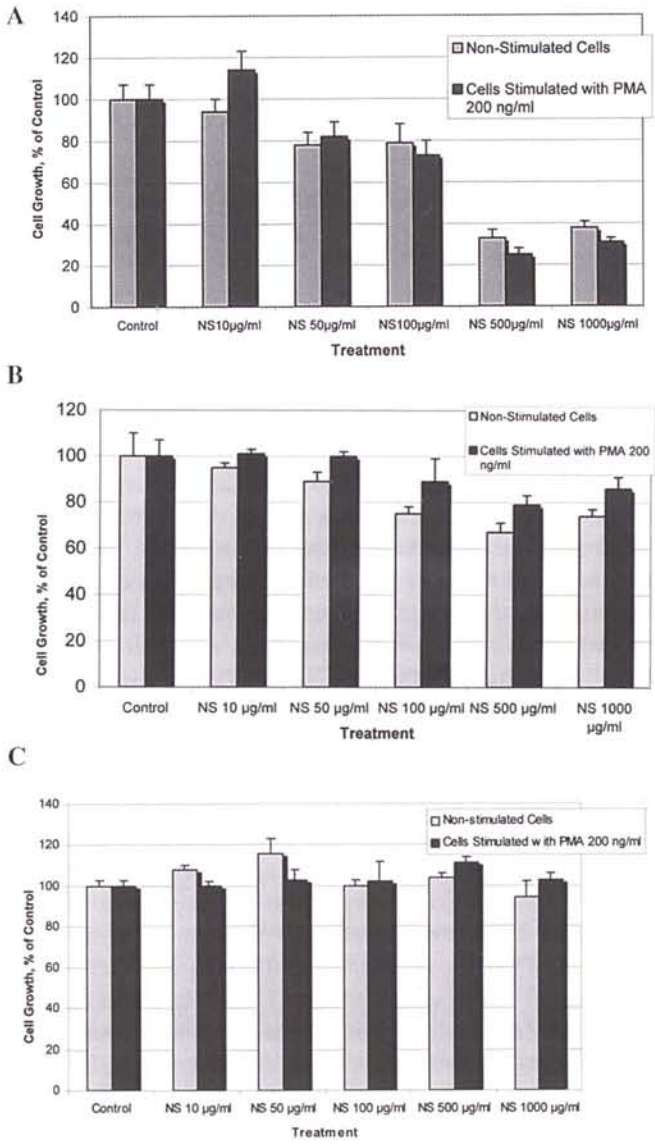

Figure 1. (A) Effect of the nutrient mixture (NS) and PMA on proliferation of human osteosarcoma cells U-2OS (p=0.006). (B) Effect of the NS and PMA on the proliferation of human osteosarcoma cells MNNG-HOS (p=0.2). (C) Effect of the NS and PMA on proliferation of human Ewing's sarcoma cells SK-ES-1 (p=0.3).

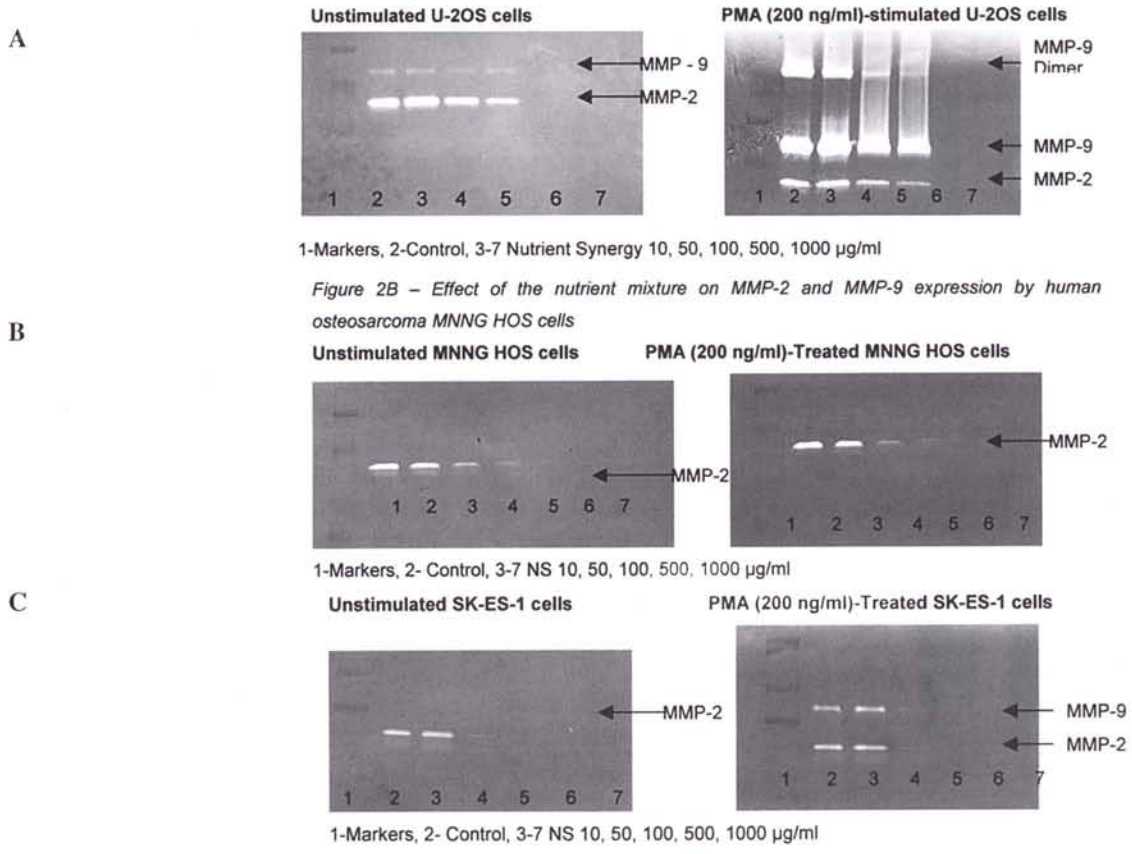

Figure 2. (A) Effect of the nutrient mixture (NS) on MMP-2 and MMP-9 expression by human osteosarcoma U-2OS cells. (B) Effect of the NS on MMP-2 and MMP-9 expression by human osteosarcoma MNNG HOS cells. (C) Effect of the nutrient mixture on MMP-2 and MMP-9 expression by human Ewing's sarcoma SK-ES-1 cells.

Plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 h. After incubation, media from the wells were withdrawn. Cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. 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.

VEGF assay. Conditioned media were collected after confluent cell culture incubation for 24 h in serum-free medium with the indicated supplements. Triplicate samples were pooled, and the level of VEGF was measured in duplicate using immunoassay kit (BioSource International) according to manufacturer's protocol. Values are expressed as mean ± range of two replicates in percentage units of unstimulated control. Unstimulated control VEGF content was 8.49±0.29 pg/ml.

Composition of the nutrient formulation. 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; and manganese 1 mg.

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

Results

Osteosarcoma proliferation study. The nutrient mixture had no significant anti-proliferative effect on human osteosarcoma U-2 OS at 10 µg/ml, but exhibited inhibition of proliferation with a maximum of 67% over the control at 500 µg/ml in unstimulated cells and 75% over control in PMA-stimulated cells, as shown in Fig. 1A. Results were statistically significant at 500 µg/ml (p=0.006). NS showed no significant effect on MNNG-HOS (Fig. 1B) or Ewing's sarcoma SK-ES-1 (Fig. 1C) untreated or PMA-induced cells.

Gelatinase zymography study. As shown in Fig. 2A, zymography demonstrated expression of MMP-2 and MMP-9 by human osteosarcoma U-2OS cells with significantly increased MMP-9 expression in PMA (200 ng/ml) treated osteosarcoma cells. Nutrient synergy inhibited the expression of both MMPs in a dose-dependent fashion with virtual total inhibition at 500 µg/ml concentration. In the MNNG-HOS cell line, only MMP-2 expression was seen, with total virtual inhibition at 500 µg/ml (Fig. 2B). Uninduced Ewing's sarcoma cells expressed MMP-2 and PMA-treated cells secreted MMP-2 and -9, with total inhibition of both at 50 µg/ml (Fig. 2C).

Invasion study. Invasion of U-20S osteosarcoma cells through Matrigel was reduced by 74% at 50 µg/ml and totally inhibited at 100 µg/ml (p=0.003) (Fig. 3). Invasion of MNNG-HOS osteosarcoma cells through Matrigel was dramatically reduced

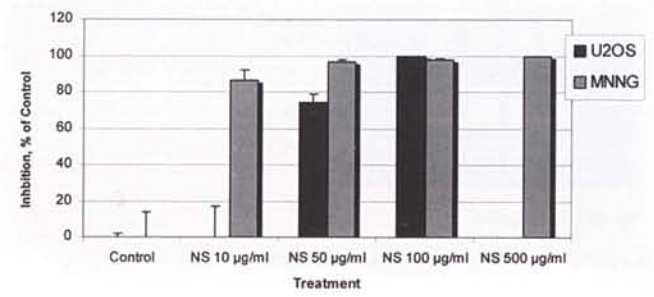

Figure 3. Effect of nutrient synergy on Matrigel invasion and migration by human osteosarcoma cells U-2OS and MNNG.

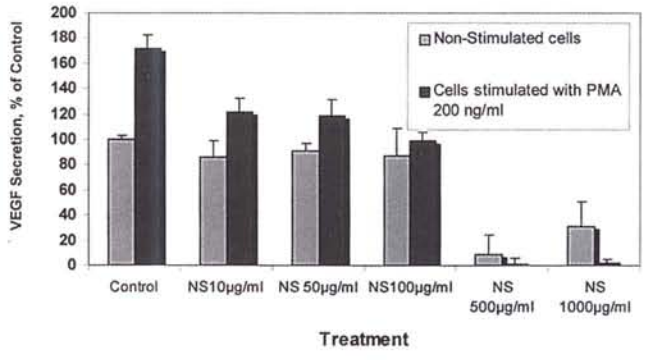

Figure 4. Effect of the nutrient mixture (NS) on VEGF expression of osteosarcoma U-2OS.

at 10 µg/ml (87%), 97% at 50 µg/ml and totally inhibited at 500 µg/ml (p=0.0002) (Fig. 3). Ewing's sarcoma cells were not invasive through Matrigel.

VEGF expression. PMA increased VEGF expression of osteosarcoma U-2OS cells in the absence of NS (up to 170% of the control) and also in the presence of low doses of NS (up to 100 µg/ml). Nutrient synergy significantly (p=0.002) reduced VEGF expression of stimulated osteosarcoma cells to 0% at NS 500 and 1000 µg/ml (Fig. 4).

Discussion

The results of this study showed dramatic and significant inhibition of matrix invasion in human osteosarcoma cell lines U-2OS and MNNG-HOS *in vitro* with the nutrient mixture. NS also decreased expressions of MMP-2 and MMP-9 in these osteosarcoma cancer cells in a dose-dependent fashion, which correlated with complete inhibition of invasion. The nutrient mixture also inhibited Ewing's sarcoma cell MMP expression.

Matrix invasion can be controlled by inhibition of MMP expression as well as by increasing connective tissue strength and stability, which contributes to 'encapsulation' of the tumor. In this study, the dose-dependent inhibitory effect of the nutrient formulation on MMP-9 and MMP-2 expression of the osteosarcoma cells was consistent with its dose-dependent inhibition of matrix invasion. In addition, matrix invasion was also modulated by enhanced stability and strength of the connective tissue 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.

Furthermore, progressive growth of neoplasms depends upon development of new blood vessels. VEGF has been reported to be an important angiogenesis factor in osteosarcoma (7) and its expression is reported to be predictive of pulmonary metastasis and poor prognosis. Early relapse and distant metastasis was reported in patients after resection of the primary tumor. A study on the effect of resection on the primary tumor in animal osteosarcoma models revealed enhanced progression of pulmonary metastasis and decreased concentration of angiogenesis inhibitor, endostatin (3). In our study, VEGF expression by human osteosarcoma cells U-2 OS, which was increased by 170% of control with PMA treatment, was completely inhibited at 500 µg/ml of the nutrient formulation.

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 (8-10); in addition, low levels of ascorbic acid have been reported in cancer patients (11-13). EGCG is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines (14-16).

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). Furthermore, in contrast to chemotherapy which causes indiscriminate cellular and ECM damage, morphological studies showed that the osteosarcoma cells were not affected even at the highest concentrations of the nutrient mixture, demonstrating that this formulation is safe for cells.

Our results suggest that this combination of nutrients has great potential for therapeutic use in the treatment of the highly aggressive osteosarcoma cancer, by inhibiting critical steps in cancer development and growth, such as cell proliferation, MMP expression, invasion, and angiogenesis.

Acknowledgements

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