Original Article

Naturally Produced Extracellular Matrix Inhibits Growth Rate and Invasiveness of Human Osteosarcoma Cancer Cells

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Abstract

Enhanced supplementation with certain naturally occurring nutrients and vitamins has been associated with a reduction in occurrence and progression of human cancer. The exact mechanisms of this action are still under investigation. Extracellular matrix (ECM) plays a key role in the development of cancer. Therefore, we studied whether nutrients could exert anticancer effects through alteration of ECM biological properties. Confluent cultures of normal human dermal fibroblasts were allowed to produce and deposit ECM during a 7 d incubation period in the presence of tested compounds. Subsequently, fibroblasts were removed and the growth rates of freshly placed cultures of human osteosarcoma cells (lines U2-OS, MMNG, or SK.ES1) on pre-formed ECM were assayed in plain cell growth medium. In addition, ECM was deposited by fibroblasts on the upper surface of a porous plastic membrane and the subsequent migration of osteosarcoma cells to the other side was assayed in non-supplemented medium. The results demonstrated that the ECM produced by normal fibroblasts treated with a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea polyphenols significantly reduced the growth rate and invasive activity of osteosarcoma cells in contrast to the non-supplemented control. The changes in ECM properties were accompanied by significant changes in ECM protein and glycosaminoglycan composition as assayed immunochemically. We conclude that anticancer effects of nutrients involve beneficial changes in ECM biological properties. The role of ECM components in ECM-dependent regulation of tumor cell activities is discussed.

Key Words: Osteosarcoma; ECM; proliferation; invasion; collagen; green tea extract; ascorbic acid; MMP-9.

Introduction

Osteosarcoma, the most common bone cancer and the sixth most common cancer in children, demonstrates aggressive, rapid growth and metastasis. Approximately 20% of children diagnosed with osteosarcoma have an advanced stage of osteosarcoma that has metastasized to the lungs, brain, and other bones *(1)*. 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.

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 *(2)* 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.

MMPs and constituents of ECM play a critical role in neoplastic invasion and metastasis. In previous studies, a unique combination of nutrients (NM) demonstrated anti invasive effects on osteosarcoma cell lines MNNG-HOS, U-2OS, and Ewings sarcoma in vitro *(3)*. We followed this with an in vivo investigation of the effect of a diet supplemented with NM (0.5%) on the growth of tumors induced by implanting human osteosarcoma MNNG xenografts in athymic nude mice *(4)*. We found that not only did NM inhibit the growth of tumors in nude mice, but that histological evaluation revealed decreased mitotic index, MMP-9, and VEGF secretion in the supplemented tumor tissues when compared to control group tissues. Structural changes in the ECM are necessary for cell migration during tissue remodeling. Recent studies have investigated the role of cell surface integrins interacting with tumor cells, mediating migration and invasion of these cells *(5)*. It was demonstrated that structure and composition of the ECM affect tumor cell adhesion, motility, and invasion through the ECM. This prompted us to investigate if NM had any effect on ECM composition and biological properties.

Methods

Cultured Cells and Materials

Normal human dermal fibroblasts were supplied by ATCC, maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and used in the experiments at passages 10th to 12th. Human osteosarcoma cells, lines SK-ES1, U2OS, and MNNG, were supplied by ATCC and maintained in 5% FBS-supplemented MEM. All cell culture reagents were from Gibco and ATCC, cell culture plastic ware was from Becton Dickinson, nutrient mixture and green tea extract (80% phenolics) were produced and supplied by Vitatech (Hayward, CA). All other reagents were from Sigma or as specified.

Osteosarcoma Cell Growth

Osteosarcoma cell proliferation was assayed by [³H]thymidine incorporation into cellular genetic

material. Cells were plated in 24-well plates at a density of 10,000 cells per cm^2 in 0.5 mL of MEM supplemented with 2% FBS. The attached cells were supplied every 24 h with fresh growth medium plus additions, as specified in the protocols. Test agents included the nutrient mixture (NM) and individual components. A stock solution of the nutrient mixture was prepared daily immediately before addition to cell cultures by solving in MEM to a concentration of 10 mg/mL, vigorously vortexed for 1 min, and filtered through a 0.2 μm sterile filter. Cell proliferation was measured 3 d later by the addition of 1 μCi/mL [³H]thymidine (MP Biomedicals) to the cell culture for the last 24 h of the experiment. Cells were washed three times with cold phosphate-buffered saline (PBS), pH 7.2, incubated with 10% trichloroacetic acid for 15 min at 4°C, washed with cold ethanol, air-dried, solubilized in 0.5 *N* sodium hydroxide, and then neutralized with hydrochloric acid. Samples were mixed with scintillation fluid and counted using a liquid scintillation counter (model 6500 LS, Beckman Instruments, USA). Cellular DNA-incorporated radioactivity was expressed as d/min per well.

Osteosarcoma Cell Growth on Fibroblast-Produced ECM

Fibroblasts were grown on collagen type I–coated culture plates to confluency in DMEM supplemented with 5% FBS. The fibroblast cultures were supplemented with NM for 8 d in the growth medium changed daily. Fibroblasts were removed from the ECM surface, exposing the fibroblast-produced ECM by treatment with 25 m*M* NH_4OH/0.5% Triton X-100 as described previously *(6)*. Osteosarcoma cells were seeded at a density of 10,000 cells per cm^2 and allowed to grow on the surface of the fibroblast-produced ECM in unsupplemented MEM/2% FBS for 3 d. Cell growth rate was measured with [³H]thymidine incorporation assay as described above.

Osteosarcoma Invasion Through Fibroblast-Produced ECM

Normal human dermal fibroblasts were plated and grown to confluency onto plastic porous (3 μm pores) membranes covered with collagen type I gel. Cells were then supplied with NM in DMEM/5% FBS for 7 d to allow for production of and deposition of extracellular matrix, as described above. Human osteosarcoma cells were grown in a culture flask in

the presence of 0.5 µCi/ml [^{3}H] thymidine for 24 h (label incorporated into cellular DNA). Cancer cells were then washed of non-incorporated label, suspended in serum-free cell culture medium, and plated onto the fibroblast extracellular matrix layer in the absence of NM. Cancer cell invasion through the porous membrane covered with cell-ECM layer was stimulated by placing 5% FBS in the lower chamber. After 24 h incubation, the number of invaded cancer cells was estimated by measuring radioactivity on the other side of membrane.

Osteosarcoma Attachment to Various Surfaces

For coincubation attachment assay, metabolically prelabeled MNNG and U2OS cells (as described above) were seeded on plastic, collagen type I or fibroblast-produced ECM at the density of 10,000 cells/cm^2 and incubated for 30 min at 37°C in serum-free medium supplemented with 250 µM ascorbate, 250 µg/mL NM, or none. Cell attachment was estimated according to surface-bound radioactivity recovered after unbound cell removal by washing with PBS. For preincubation attachment assay, MNNG cells were pre-incubated for 3 d with 250 µM ascorbate or 250 µg/mL NM or control media. Cell attachment to collagen I substrate was assayed in plain serum-free medium, as described above.

Determination of ECM Composition

Human dermal fibroblasts were seeded in a 96-well plate covered with collagen type I in 5% FBS/DMEM. After reaching confluence cells were supplemented with 100 µg/mL nutrient mixture or corresponding amounts of ascorbic acid (100 µM) or green tea extract (20 µg/mL) for 8 d with media change every other day. Extracellular matrix was exposed by cell removal with NH$_4$OH/Triton X-100 treatment as described before. Sandwich-type immunoassay was applied to determine changes in component accumulation in exposed ECM. Primary antibodies were rabbit polyclonal for collagen type IV, fibronectin and laminin (Rockland Immuno-chemicals), monoclonal for collagen type I, elastin, chondroitin sulfate (Sigma), and heparan sulfate (Chemicon), and biotin-conjugated hyaluronic acid binding protein (US Biological) was used for hyaluronic acid assay. Primary antibody was incubated with ECM for 2 h at room temperature in 1% bovine serum albumin (BSA) in PBS followed by

washing with 0.1% BSA/PBS and incubation with corresponding secondary antibody labeled with horseradish peroxidase (all from Rockland) for 1.5 h at room temperature. Streptovidin–peroxidase conjugate (Rockland) was used for hyaluronic acid assay. TMB substrate (Rockland) was used for detecting ECM-bound peroxidase after second washing cycle. Data are presented as average optical density values at 450 nm for four repetitions after correction for cell-free control and expressed as percentage to supplementation-free controls.

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 µg; copper 2 mg; manganese 1 mg.

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 (7). Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression (8). N-acetyl cysteine has been observed to inhibit MMP-9 activity (9) and invasive activities of tumor cells (10). Selenium has been shown to interfere with MMP secretion and tumor invasion (11), as well as migration of endothelial cells through ECM (10). 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 (12).

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 *(13)*. 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. Correlation coefficients were determined using MedCalc Software (Mariakerke, Belgium).

Results

Effect of the Nutrient Mixture on Osteosarcoma Cell Growth

NM significantly inhibited osteosarcoma cell growth. In SK-ES1 cells, proliferation was reduced compared to the control by 85.7% ($p = 0.0002$) at 25 μg/mL NM, by 95.8% ($p < 0.0001$) at 50 μg/mL NM, and by 96.2% ($p < 0.0001$) at 100 μg/mL NM (Fig. 1A). Similarly, in U2OS cells, NM reduced proliferation over the control by 49% ($p = 0.0067$) at 10 μg/mL NM, 84% ($p = 0.0005$) at 25 μg/mL NM, and 99.4% ($p = 0.0002$) at 100 μg/mL NM (Fig. 1B). With MNNG-HOS cells, dose-dependent inhibition was also seen; antiproliferative effect of NM was 22.4% ($p = 0.03$) at 25 μg/mL, 40% ($p = 0.003$) at 50 μg/mL, and 66.9% ($p = 0.0002$) at 100 μg/mL concentration (Fig. 1C).

Effect of ECM-Mediated Effects of NM on Osteosarcoma Growth

The ECM produced by normal fibroblasts treated with NM 100 μg/mL reduced the growth rate of osteosarcoma cells in contrast to the non-supplemented control. Although the results were not statistically significant, MNNG osteosarcoma cell growth (Fig. 2A) was reduced by 11.6% ($p = 0.5$) and U2OS osteosarcoma cell growth (Fig. 2B) by 15.2% ($p = 0.38$). ECM-mediated reduction of SK.ES-1 osteosarcoma cell growth (Fig. 2C) reached statistical significance (17.8%, $p = 0.037$).
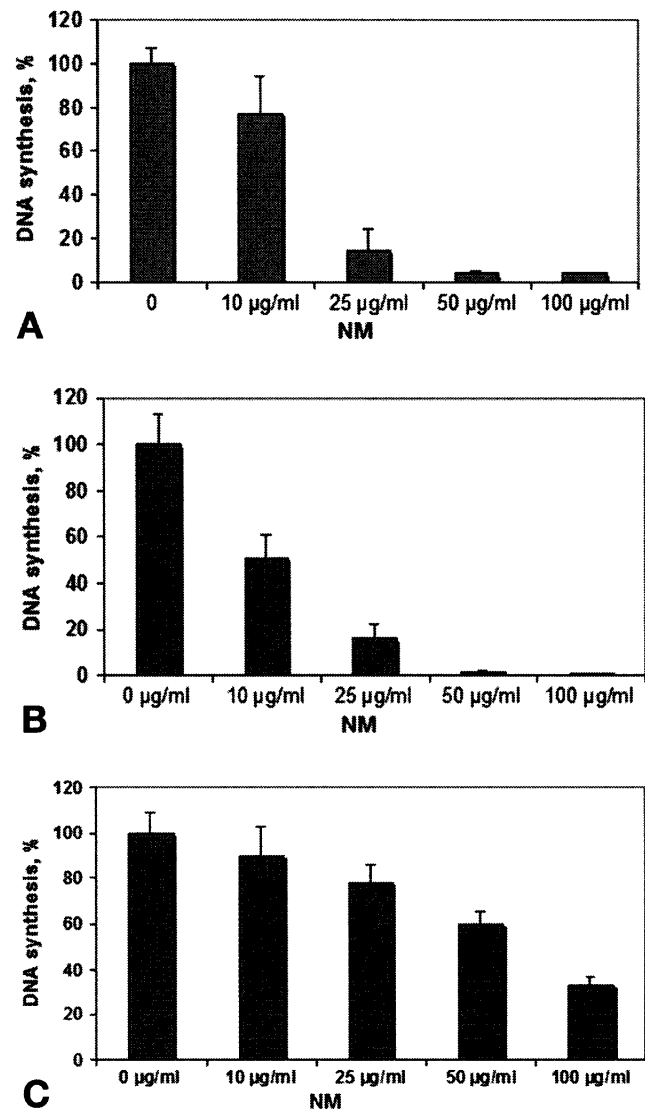

Fig. 1. Effect of NM on human osteosarcoma cell growth. Proliferating osteosarcoma cultures were incubated with indicated amounts of the nutrient mixture for 72 h. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA during last 24 h of the experiment; **(A)** SK.ES-1 osteosarcoma cell growth; **(B)** U2OS osteosarcoma cell growth; **(C)** MNNG-HOS osteosarcoma cell growth.

Osteosarcoma Invasion Through Fibroblast (FB)-produced ECM

The ECM produced by normal fibroblasts treated with the nutrient mixture significantly reduced the invasion of osteosarcoma cells in a dose-dependent
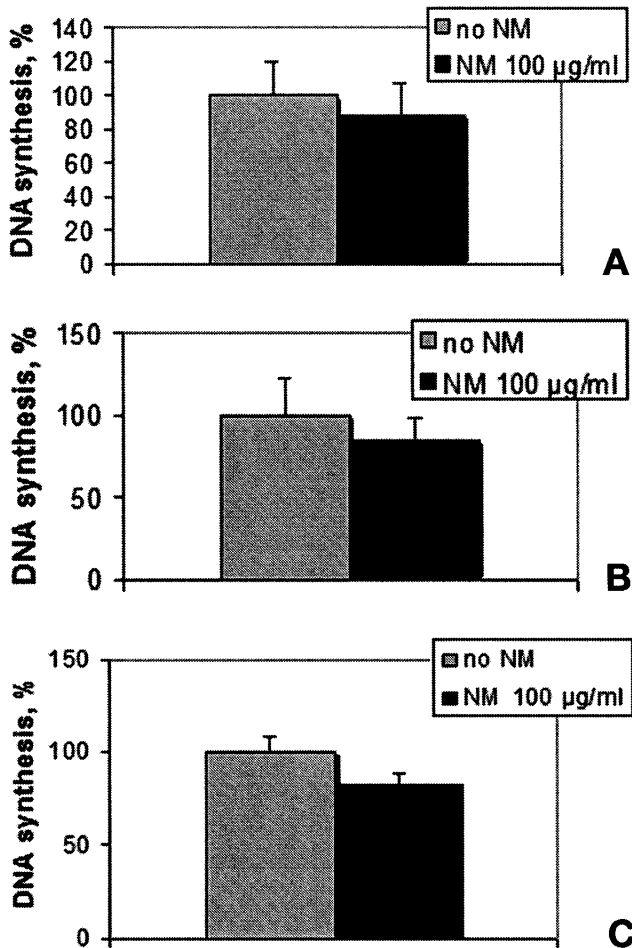
Fig. 2. Effects of NM on osteosarcoma cell growth on HDF-produced ECM. ECM production for 8 days, osteosarcoma cell growth 72h. Cell growth rate was evaluated by incorporation of [^{3}H]thymidine into cellular DNA during last 24 h of the experiment; **(A)** MNNG osteosarcoma cell growth; **(B)** U2OS osteosarcoma cell growth; **(C)** SK.ES-1 osteosarcoma cell growth.

Fig. 3. Effect of NM on U2OS cell invasion through fibroblast-produced ECM. U2OS cells were plated on top of fibroblast-produced ECM. Cell migration was initiated by addition of 10% FBS to the lower chamber. Cell invasion was estimated in 24 h by counting cells migrated to the other side of the membrane and expressed as % of unsupplemented control

Fig. 4. U2OS cell attachment to different surfaces in control media.

manner ($r = -0.628$, $p = 0.029$), with 33% inhibition at 100 μg/mL NM, as shown in Fig. 3.

Effects of NM on Osteosarcoma Cell Attachment to Various Substrates

Osteosarcoma U2OS attachment to collagen was greater than to plastic (by 35%, $p = 0.02$) and greater still to Matrigel than to plastic (by 128%, $p = 0.0006$), as shown in Fig. 4. Coincubation with ascorbate decreased osteosarcoma U2OS cell attachment to plastic by 25% ($p = 0.05$), as shown in Fig.
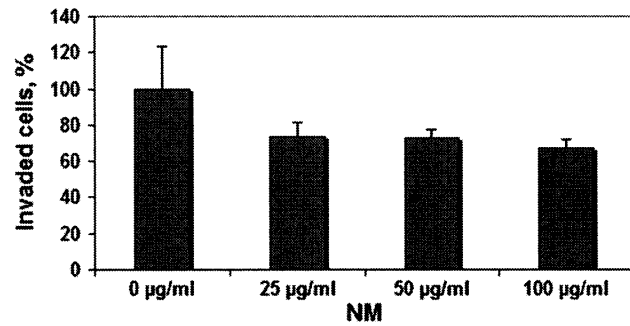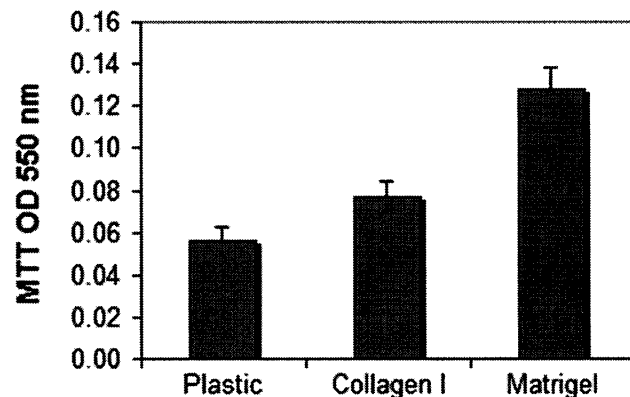
5. Coincubation with NM further decreased U2OS cell attachment by 61% ($p = 0.001$). Similarly, coincubation with ascorbate and NM reduced osteosarcoma U2OS cell attachment to collagen I, as shown in Fig. 6. Coincubation with ascorbate 250 μM resulted in 17.7% ($p = 0.04$) decreased osteosarcoma MNNG-HOS cell attachment to collagen I; furthermore, coincubation with NM 250 μg/mL resulted in 46.6% ($p = 0.0001$) inhibition of osteosarcoma MNNG-HOS cell attachment to collagen I (Fig. 7). Preincubation of osteosarcoma MNNG-HOS cells
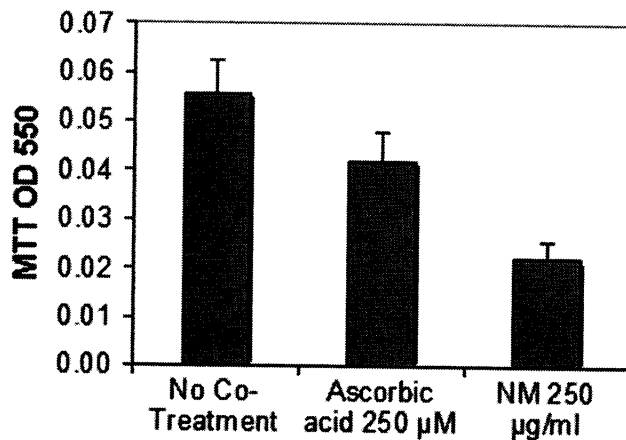
Fig. 5. U2OS cell attachment to plastic in the presence of ascorbic acid, NM or control.
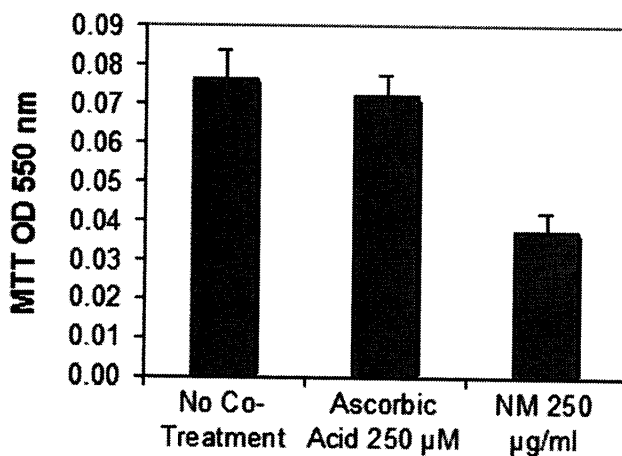

Fig. 6. Effect of co-treatment with NM or ascorbic acid on U2OS cell attachment to collagen I.

Fig. 7. Effect of co-treatment with NM or ascorbic acid on MNNG cell attachment to collagen I.

Fig. 8. Effects of pre-incubation with 250 μ*M* ascorbic acid or 250 μg/mL NM on MNNG HOS osteosarcoma cell attachment to collagen I.

with ascorbic acid had no significant effect on collagen I attachment. However, pre-treatment with NM 250 μg/mL resulted in significant reduction (57.9%, $p = 0.0002$) in osteosarcoma cell attachment to collagen I (Fig. 8). Significant correlation was observed between fibroblast-mediated extracellular matrix components, as shown in Table 1.

Effects of the Nutrients on Fibroblast-Produced ECM Composition

Incorporation of collagen types I and IV into the ECM by fibroblasts was dramatically increased over that in the control ECM with exposure to ascorbic acid (1315% and 389%, respectively), but reduced
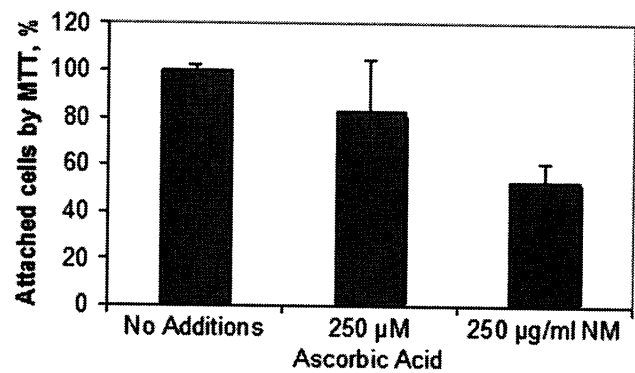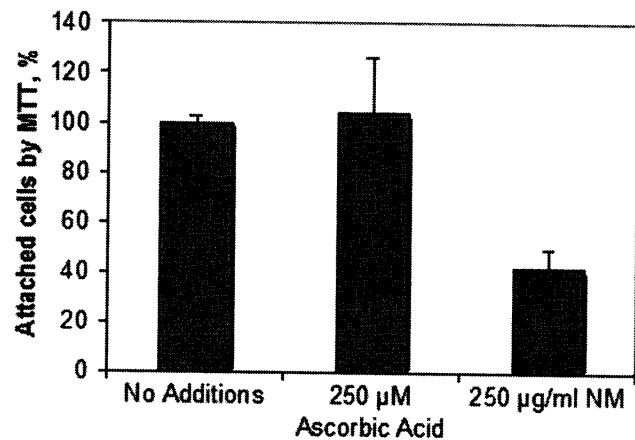
significantly with exposure to the nutrient mixture (by 37% for collagen I and 80% for collagen IV), as shown in Table 2. Exposure of fibroblasts to the nutrient mixture resulted in greater inhibition of incorporation of both collagens into the fibroblast-mediated ECM than did exposure of fibroblasts to green tea extract alone, which resulted in 25% reduction of collagen type I and 72% reduction of collagen type IV when compared to the control ECM. Elastin and heparan sulfate incorporation into the ECM exposed to 100 μg/mL of the nutrient mixture decreased significantly over the control ECM, by 51% and 68%, respectively. Elastin was not significantly affected by ascorbic acid and green tea extract. Heparan sulfate

Table 1
Correlation Between Fibroblast-Mediated Extracellular Matrix Components

	Collagen type I	Collagen type IV	Heparan sulfate
Collagen type IV	$r = 0.9856$ $p = 0.0144$		
Heparan sulfate	$r = 0.9611$ $p = 0.0389$	$r = 0.9926$ $p = 0.0074$	
Hyaluronic acid	$r = 0.9858$ $p = 0.0020$	$r = 0.9712$ $p = 0.0058$	$r = 0.9607$ $p = 0.0093$

Table 2
Effect of Nutrients on Composition of Fibroblast-Mediated Extracellular Matrix

	No additions	NM 100 µg/mL	Ascorbic Acid 100 µM	Green Tea Extract 20 µg/mL
Collagen type I	100.0 (15.5)	63.3 (12.5)*	1315.2 (164.4)**	75.0 (22.6)
Collagen type IV	100.0 (17.2)	20.4 (13.9)**	389.2 (35.8)**	28.3 (5.6)**
Fibronectin	100.0 (11.3)	90.0 (5.9)	114.3 (19.5)	116.7 (17.3)
Laminin	100.0 (5.2)	88.9 (5.3)*	93.2 (11.5)	95.0 (11.8)
Elastin	100.0 (17.9)	49.0 (8.9)**	104.4 (27.2)	86.3 (33.7)
Chondroitin Sulfate	100.0 (5.7)	99.6 (7.5)	118.0 (15.7)	149.7 (4.9)**
Heparan Sulfate	100.0 (22.1)	32.2 (16.2)**	291.5 (21.6)**	11.5 (20.8)**
Hyaluronic Acid	100.0 (12.2)	108.5 (16.0)	238.5 (78.9)*	79.5 (9.9)

*$p < 0.05$; **$p < 0.01$.

was significantly increased in the presence of ascorbic acid and significantly decreased in the presence of green tea extract. However, the nutrient mixture did not significantly affect fibronectin, chondroitin sulfate, and hyaluronic acid incorporation.

Discussion

The interaction of neoplastic cells with the underlying extracellular matrix is a central component of cancer development, including tumor growth and ECM degradation by proteases leading to invasion and angiogenesis. In this study we investigated whether nutrients could exert anticancer effects through alteration of ECM biological properties. The results demonstrated that the ECM produced by normal fibroblasts treated with a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea polyphenols significantly reduced the growth rate and invasive activity of osteosarcoma cells in contrast to the non-supplemented control. Tumor cell adhesion to the ECM also plays a part in mod-

ulating cancer progression. We demonstrated that coincubation, as well as preincubation of osteosarcoma cells with the nutrient mixture, inhibited adhesion of osteosarcoma cells to various substrates, including collagen type I.

Matrix invasion can be controlled by inhibition of MMP activity, as well as by strengthening and stabilizing the connective tissue. Many cancers often demonstrate fragmentary basement membranes in contrast to the continuous basement membrane found in their benign counterparts *(14)*. Thus, a reduced capacity of cancer cells to synthesize an optimal intact basement membrane may also play a role in tumor cell invasion. The change in ECM composition with cancer progression has not been studied in depth, and there are conflicting results with matrix proteins associated with cancer. Some gastrointestinal cancer cell lines were found to be unable to produce laminin *(15)*, while in malignant melanoma patients, laminin, hyaluronan, tenascin-C, and collagen type IV levels were seen to increase over that found in healthy individuals *(16)*.

In our study, exposure of fibroblasts to the nutrient mixture resulted in an ECM with significantly decreased collagen, elastin, and heparan sulfate levels, compared to control ECM, resulting in increased stability of the ECM, as evidenced by inhibition of osteosarcoma cells through the fibroblast-produced ECM. Thus, the ECM strength in inhibiting invasion was increased with exposure to NM, with a corresponding decrease in production of matrix proteins, suggesting that the inhibition was not due to mechanical blocking secondary to increased ECM thickness, but due to other mechanisms, such as decreasing susceptibility to integrin interaction with the ECM. For example, the significant reduction of laminin, through which integrins are reported to bind, would lead to fewer binding sites. In addition, the significant reduction in production of heparan sulfate, which has been shown to be a binding agent for growth factors, would result in reduction of storage of growth factors available for cell growth stimulation, and thus would explain the reduction observed in osteosarcoma cell proliferation in cells exposed to NM. As mentioned previously, in earlier studies NM demonstrated anti invasive effects on osteosarcoma cell lines MNNG-HOS, U-2OS, and Ewing's sarcoma in vitro *(3)*, as well as in vivo, where diet supplementation with NM (0.5%) significantly inhibited the growth of tumors induced by implanting human osteosarcoma MNNG xenografts in athymic nude mice *(4)*.

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 possible in various pathological stages and through deficient diets. In addition to suppressing tumor growth and MMP secretion, green tea extract components have also been shown to increase cell stability and decrease cell adhesion, cell spreading, and protease binding *(17,18)*.

Conclusion

The ECM synthesized by normal fibroblasts treated with NM demonstrated increased stability and significantly reduced the osteosarcoma cell growth rate, invasive activity, and adhesion to collagen I and other substrates. While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of this study and our previous studies on osteosarcoma suggest the nutrient mixture containing lysine, proline, arginine, ascorbic acid, and green tea extract has potential therapeutic use in the treatment of the highly aggressive osteosarcoma cancer, by beneficially modulating ECM biological properties, suppressing tumor growth independent of immune system function, and inhibiting critical steps in cancer metastasis, such as MMP secretion and invasion.

References

1. Miller R, Dowshen S, Trigg M: Childhood Cancer:osteosarcoma. The Nemours Foundation: Kids Health http://kidshealth.org/parent/medical/cancer/cancer_osteosarcoma_p1-3.htmL, May 2002.
2. Rath M, Pauling L. Plasmin-induced proteolysis and the role of apoprotein(a), lysine and synthetic analogs. *Orthomoloecular Medicine* 1992; **7**:17–23.
3. Roomi MW, Ivanov V, Kalinovsky T, Niedzeicki A, Rath M. Antitumor effect of nutrient synergy on human osteosarcoma cells U-2OS, MNNG-HOS and Ewing's sarcoma SK-ES.1. *Oncol Rep* 2005; **13**:253–257.
4. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. 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. *Med Oncol* 2006; **23**:411–418.
5. Uhm JH, Gladson CL, Rao JS. The role of integrins in the malignant phenotype of gliomas. *Fronti Biosci* 1999; **4**:d188–199.
6. Ivanov VO, Ivanova SV, Niedzwiecki A. Ascorbate affects proliferation of guinea-pig vascular smooth muscle cells by direct and extracellular matrix-mediated effects. *J Mol Cell Cardiol* 1997; **29**(12):3293–3303.
7. Kawakami S, Kageyama Y, Fujii, Kihara K, Oshima H. Inhibitory effects of N-acetyl cysteine on invasion and MMP 9 production of T24 human bladder cancer cells. *Anticancer Res* 2001; **21**:213–219.
8. Morini M, et al. The role of the thiol N-acetyl cysteine in the prevention of tumor invasion and angiogenesis. *Int J Biol Markers* 1999; **14**:268–271.
9. Yoon SO, Kim MM, Chung AS. Inhibitory effects of selenite on invasion of HT 1080 tumor cells. *J Biol Chem* 2001; **276**:20085–20092.
10. Maramag C, Menon M, Balaji KC, Reddy PG, Laxmanan S. Effect of vitamin C on prostate cancer cells in vitro: effect on cell number, viability and DNA synthesis. *Prostate* 1997; **32**:188–195.

11. Hare Y. *Green Tea: Health Benefits and Applications,* Marcel Dekker: New York, 2001.

12. Cooke JP, Dzau VJ. Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med* 1997; **48:**489–509.

13. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Synergistic antitumor effect of ascorbic acid, lysine, proline, and epigallocatechin gallate on human fibrosarcoma cells HT-1080. *Ann Cancer Res Ther* 2004; **12:**1–2.

14. Barsky SH, Siegel GP, Jannotta F, Liotta LA. Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab Invest* 1983; **49:**140–147.

15. Akashi T, et al. Synthesis of basement membrane by gastrointestinal cancer cell lines. *J Pathol* 1999; **187:**223–228.

16. Burchardt ER, Hein R, Bosserhoff AK. Laminin, hyaluronan, tenascin-C and type VI collagen levels in sera from patients with malignant melanoma. *Clin Exp Dermatol* 2003; **28**(5):515–520.

17. Weyant MJ, Carothers AM, Dannenberg AJ, Bertagnolli M. (+)-catechin inhibits intestinal tumor formation and suppresses focal adhesion kinase activation in the Min/+ mouse. *Cancer Res* 2001; **61:**118–125.

18. Bracke ME, Van Cauwenberge RM, Mareel MM. (+)-catechin inhibits the invasion of malignant fibrosarcoma cells into chick heart in vitro. *Clin Exp Metastasis* 1984; **2**(2):161–170.