

Original Article

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

V. Ivanov, S. Ivanova, M.W. Roomi, T. Kalinovsky, A. Niedzwiecki, and M. Rath

Dr. Rath Research Institute, Cancer Division, 1260 Memorex Drive, Santa Clara, CA 95050

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

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Aleksandra Niedzwiecki, 1260 Memorex Drive, Santa Clara,
CA 95050. E-mail: a.niedz@drath.com

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 cm² 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 mM NH₄OH/0.5% Triton X-100 as described previously (6). Osteosarcoma cells were seeded at a density of 10,000 cells per cm² 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 $\mu\text{Ci/ml}$ [^3H] 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/cm² and incubated for 30 min at 37°C in serum-free medium supplemented with 250 μM ascorbate, 250 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$ nutrient mixture or corresponding amounts of ascorbic acid (100 μM) or green tea extract (20 $\mu\text{g/mL}$) for 8 d with media change every other day. Extracellular matrix was exposed by cell removal with NH_4OH /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 Immunochemicals), 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. Streptavidin-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 $\mu\text{g}/\text{mL}$ EGCG to that of 50 $\mu\text{g}/\text{mL}$ (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 \pm 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 $\mu\text{g}/\text{mL}$ NM, by 95.8% ($p < 0.0001$) at 50 $\mu\text{g}/\text{mL}$ NM, and by 96.2% ($p < 0.0001$) at 100 $\mu\text{g}/\text{mL}$ NM (Fig. 1A). Similarly, in U2OS cells, NM reduced proliferation over the control by 49% ($p = 0.0067$) at 10 $\mu\text{g}/\text{mL}$ NM, 84% ($p = 0.0005$) at 25 $\mu\text{g}/\text{mL}$ NM, and 99.4% ($p = 0.0002$) at 100 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$, 40% ($p = 0.003$) at 50 $\mu\text{g}/\text{mL}$, and 66.9% ($p = 0.0002$) at 100 $\mu\text{g}/\text{mL}$ concentration (Fig. 1C).

Effect of ECM-Mediated Effects of NM on Osteosarcoma Growth

The ECM produced by normal fibroblasts treated with NM 100 $\mu\text{g}/\text{mL}$ 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%, $p = 0.037$).

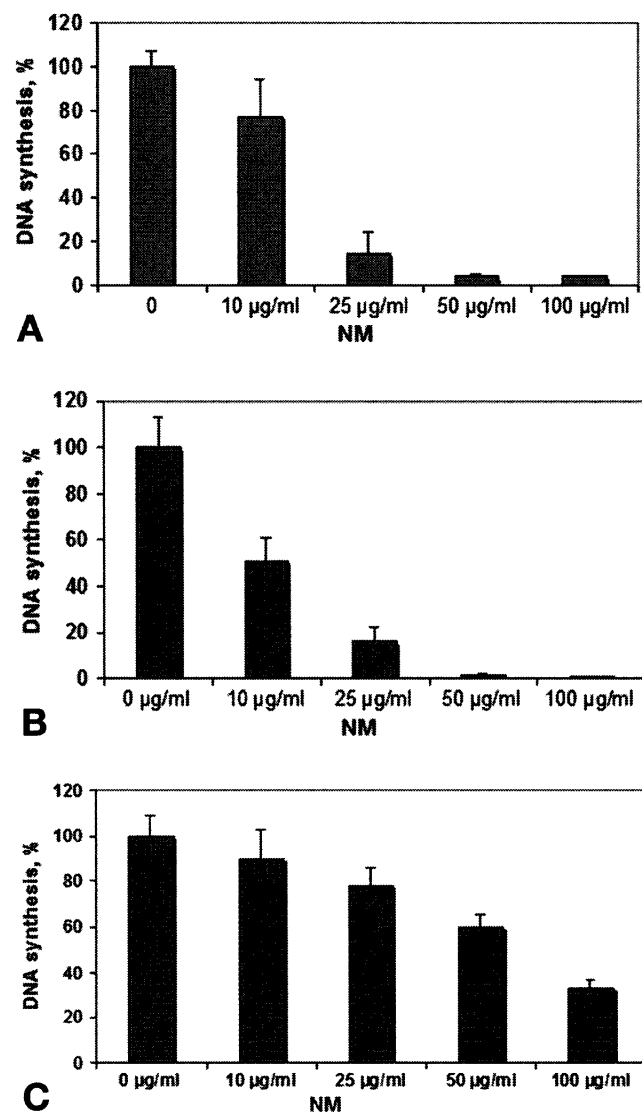


Fig. 1. Effect of NM on human osteosarcoma cell growth. Proliferating osteosarcoma cultures were incubated with indicated amounts of the nutrient mixture for 72 h. Cell growth rate was evaluated by incorporation of [^3H]-thymidine into cellular DNA during last 24 h of the experiment; (A) SK.ES-1 osteosarcoma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

