

Original Article

Inhibitory Effects of a Nutrient Mixture on Human Testicular Cancer Cell Line NT 2/DT Matrigel Invasion and MMP Activity

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Abstract

Current treatment of testicular cancer is associated with secondary malignancy, infertility, and cytotoxicity. Based on reported antimetastatic potential, we investigated the effect of a nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid, and green tea extract on human testis cancer cell line NT 2/DT by measuring cell proliferation/cytotoxicity, modulation of MMP-2 and MMP-9 secretion, and cancer cell invasive potential. Human testis cancer cells NT 2/DT (ATCC) were grown in DME medium. At near confluence, the cells were treated with NM dissolved in media and tested at 0, 10, 50, and 100 $\mu\text{g}/\text{mL}$ in triplicate at each dose. Cells were also treated with PMA 200 ng/mL to study enhanced secretion of MMP-9. Cell proliferation/cytotoxicity was evaluated by MTT assay, MMP activity by gelatinase zymography, and invasion through Matrigel. The nutrient mixture showed no significant effect on testis cancer cell growth. Zymography demonstrated secretion of MMP-2 by untreated human testis cancer cells and MMP-9 with PMA induction. NM inhibited secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-9 at 100 $\mu\text{g}/\text{mL}$. Invasion of human testis cancer cells through Matrigel was reduced by 84% at 50 $\mu\text{g}/\text{mL}$ and at 100 $\mu\text{g}/\text{mL}$ ($p = 0.004$). NM significantly inhibited MMP secretion and matrix invasion in testicular cancer cells without toxic effect, indicating potential as an anticancer agent.

Key Words: Testicular cancer; MMPs; Matrigel invasion; nutrients; green tea extract; ascorbic acid; lysine.

Introduction

The American Cancer Society estimates 8,010 new cases and 390 deaths from testicular cancer in the United States in 2005 (1). Approximately 95% of patients diagnosed with stage I nonseminoma experience complete remission (2). Although commonly used cisplatin-based combination chemotherapy (cisplatin, etoposide, and bleomycin) claims a suc-

cess in approx 80% of patients with metastatic testicular cancer (3), it is associated with numerous side effects and a substantial loss of quality of life.

Standard treatment of stage I seminoma involves removal of the testicle through a radical inguinal orchiectomy followed by radiation therapy. CT scan showing metastasis to the lymph nodes will generally be treated by a retroperitoneal lymph node dissection. A retrospective study on 124 randomly selected patients in complete remission treated at Hanover University Medical School for testicular cancer found that 20% of the patient population was unable to father children 2 yr following cure as well

Received 10/21/06; Accepted 11/17/06

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as sexual dysfunction in patients treated by retroperitoneal lymph node dissection and secondary resection of residual retroperitoneal tumor mass (4).

Although a number of studies have shown that chemotherapy can eradicate testicular cancer, this treatment is accompanied by serious side effects, such as secondary morbidities, pulmonary toxicity, cardiotoxicity, neurotoxicity, ototoxicity, vascular toxicity, infertility, and impaired sexual function (5–8). Given that testicular cancer primarily affects men ages 18–32 and that many patients either do not exercise cryopreservation of spermatozoa or are not advised of this approach by their physicians, both surgery and chemotherapy can be extremely detrimental to a patient's ability to father a child. Additionally, the reported harmful cytotoxic side effects of chemotherapy are compelling support for safer alternative treatment approaches to testicular cancer.

Tumor invasion of neighboring tissue is dependent on degradation of the extracellular matrix (ECM) components by MMPs produced by tumor and stromal cells. While remodeling of the ECM is crucial to many normal biological processes, such as wound healing and reproduction, excessive degradation of matrix components by matrix metalloproteinases (MMPs) have been implicated in invasive neoplastic diseases (9). Studies have shown that the aggressiveness of the cancer (invasiveness, grade, and stage) is highly correlated with the expression of MMPs. Rath and Pauling proposed that natural inhibitors, such as lysine and ascorbic acid, have the potential to inhibit tumor growth and expansion by modulation of ECM proteolysis and optimizing connective tissue integrity (10).

In the current study, we investigated a specific nutrient combination (NM) on human testis cancer cell line NT 2/DT by measuring cell proliferation, modulation of MMP-2 and MMP-9 secretion, and cancer cell invasive potential. The nutrient mixture (NM) was formulated based on targeting key physiological pathways involved in cancer progression and metastasis. For example, 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 (11). Green tea extract has been shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression (12). N-acetyl cysteine has been observed to inhibit MMP-9 activity (13) and invasive activities of tumor cells (14). Selenium has been shown to interfere with MMP expression and tumor invasion (15), as well as migration of endothelial cells through ECM (14). 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 (16).

Based on the evidence available in the literature and our own research, we postulated 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 (17). Thus, by including nutrients such as 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.

Methods and Materials

Cell Culture

Human testes cancer cells NT 2/DT, obtained from ATCC (American Type Culture Collection, Rockville, MD), were grown in DME medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA). 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 (NM), dissolved in media, and tested at 0, 10, 50, and 100 µg/mL in triplicate at each dose and returned to the incubator. Cells were also treated with PMA 200 ng/mL to induce MMP-9 secretion.

MTT Assay

Cell proliferation was evaluated by MTT assay, 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. This test is a good index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) and 500 μ L of MTT (Sigma cat. no. M-2128) 0.5 mg/mL in media was added to each well. 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 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 secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under nonreduced conditions. Culture media (20 μ L) mixed with sample buffer was loaded and SDS-PAGE was performed with Tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 1200 dpi. The intensity of the bands was evaluated using a pixel-based densitometer program Un-Scan-It, Version

5.1, 32-bit, by Silk Scientific Corporation (Orem, UT, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi), and expressed as a percentage of control. The R_2 value (0 to 1) on graphs represents how well the line of best fit falls on the dependent data, with $R_2 = 1.0$ being the best possible fit.

Matrigel Invasion Studies

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, human testis cancer 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 h. 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 Nutrient Mixture (NM)

Stock solution of the nutrient mixture was 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 μ g; copper 2 mg; manganese 1 mg.

Statistical Analysis

The results were expressed as means \pm SD for the groups. Data was analyzed by independent sample "t" test.

Results

Cell Proliferation Study

The nutrient mixture had no significant effect on proliferation of testis cancer cells NT 2/DT within

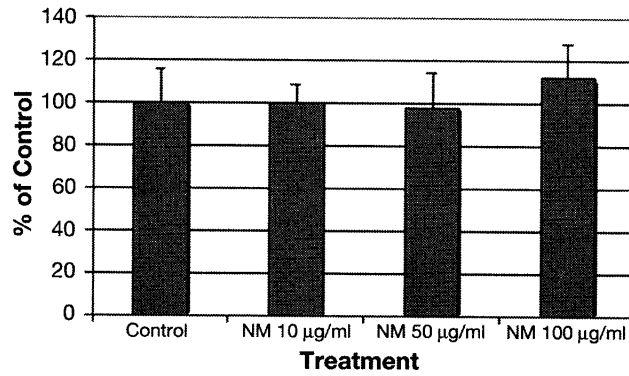


Fig. 1. Effect of the nutrient mixture (NM) on human testis cancer NT 2/DT cell proliferation: MTT assay 24 h. The nutrient mixture had no significant effect on proliferation of testis cancer cells NT 2/DT within the tested concentration range (up to 100 µg/mL).

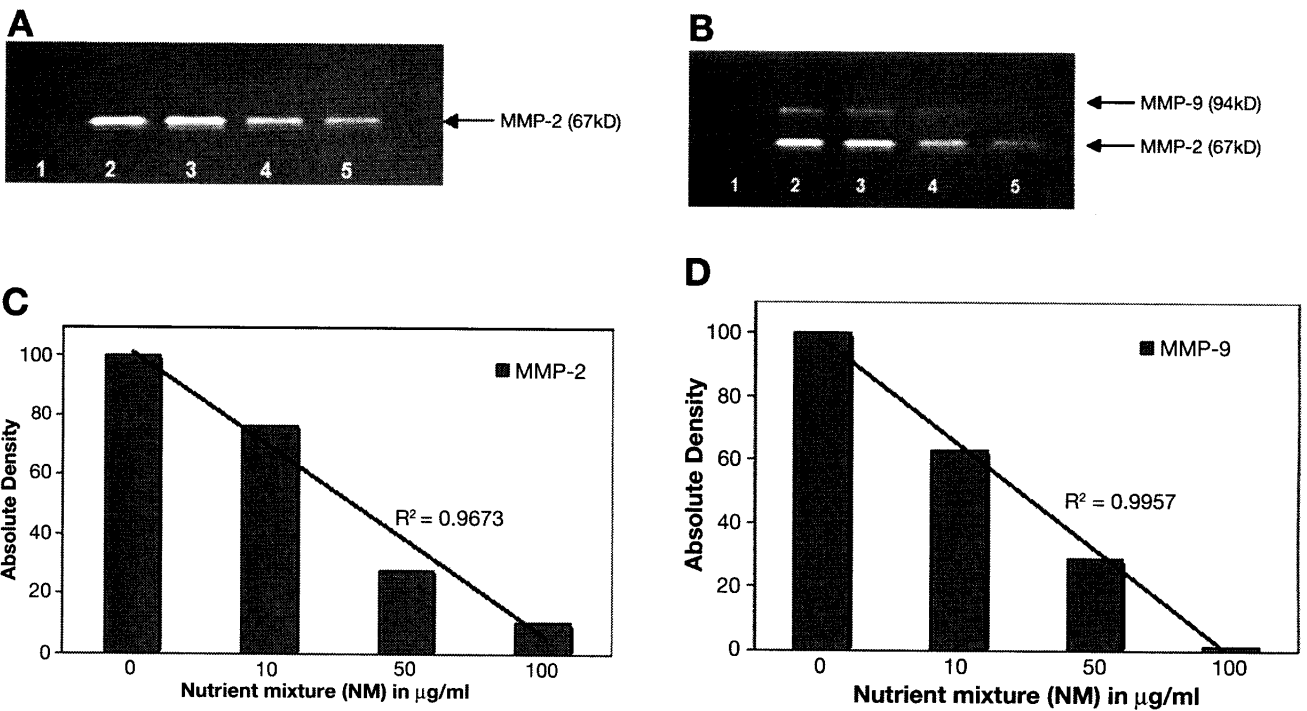


Fig. 2. Effect of the nutrient mixture (NM) on MMP-2 and MMP-9 secretion by testes cancer NT 2/DT cells. Zymogram: Uninduced cells (A) and PMA (200 ng/mL)-treated cells (B). Legend. 1, Markers, 2, Control, 3–5 NM 10, 50, 100 µg/mL. Zymography demonstrated secretion of MMP-2 and PMA-induced MMP-9 activity. The nutrient mixture (NM) inhibited secretion of both MMPs in a dose-dependent fashion with virtual total inhibition at 100 µg/mL. Densitometry Analysis. Effect of NM on relative activity of MMP-2 (C) and MMP-9 (D) in PMA (200 ng/mL)-treated testis cancer cells NT 2/DT.

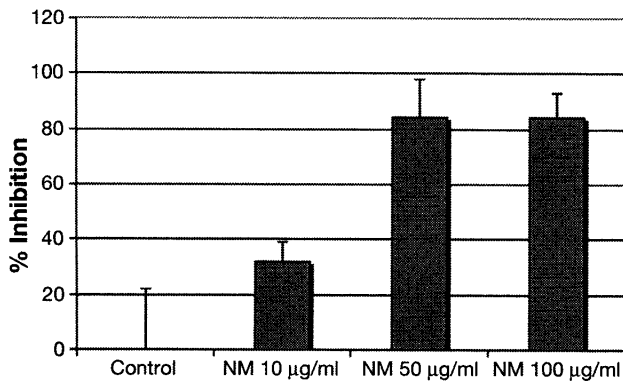


Fig. 3. Effect of the nutrient mixture (NM) on human testis cancer cell NT 2/D Matrigel invasion. The nutrient mixture (NM) significantly reduced the invasion of testis cancer cells through Matrigel in a dose-dependent fashion, with 84% inhibition at 50 µg/mL and at 100 µg/mL NM ($p < 0.004$).

the tested concentration range (up to 100 µg/mL), as shown in Fig. 1.

Gelatinase Zymography Study

Zymography demonstrated MMP-2 in untreated NT 2/DT cells (Fig. 2A) and PMA (200 ng/mL)-induced MMP-9 secretion by testis cancer cells (Fig. 2B). NM inhibited secretion of both MMPs in a dose-dependent fashion with 90% inhibition of MMP-2 and 99% inhibition of MMP-9 at 100 µg/mL, as shown in zymograms in Figs. 2A and 2B. Densitometry analysis on relative activity of MMP-2 showed 23% inhibition at 10 µg/mL and 72% at 50 µg/mL and 90% at 100 µg/mL, with $R^2 = 0.9673$ (Fig. 2C). For MMP-9, relative activity per densitometry revealed 38% inhibition at 10 µg/mL, 71% at 50 µg/mL, and 99% at 100 µg/mL, with $R^2 = 0.9957$ (Fig. 2D).

Invasion Study

The nutrient mixture (NM) significantly reduced the invasion of testis cancer cells through Matrigel in a dose-dependent fashion, with 84% inhibition at 50 µg/mL and at 100 µg/mL NM ($p < 0.004$), as shown in Fig. 3.

Discussion

Many studies have highlighted the role of matrix metalloproteinases (MMPs) in tissue remodeling

and tumor progression through degradation of extracellular matrix (ECM) components, and in angiogenesis. Inhibition of matrix metalloproteinases is a promising strategy against testicular cancer spread as metastasis depends on tumor cell breach of the ECM and the presence of adequate blood supply.

Owing to this, researchers have focused on investigating the efficacy of new treatment methods that target universal pathomechanisms involved in cancer progression, such as control of proteolytic activity of the ECM. Rath and Pauling have suggested targeting plasmin-mediated mechanisms with lysine and lysine analogs, as strengthening the surrounding connective tissue can prevent matrix invasion, contributing to the “encapsulation” of the tumor (10). Optimization of synthesis and structure of collagen fibrils depends on hydroxylation of proline and lysine residues in collagen fibrils. Adequate levels of ascorbic acid are essential for the hydroxylation of proline and lysine. Furthermore, lysine prevents cell migration by preventing collagen-digesting enzymes from binding to plasminogen-active sites, thereby blocking the activation of plasmin by plasminogen.

The dose-dependant inhibitory effect of the nutrient mixture on human testis cancer cell line NT 2/DT MMP secretion was consistent with its dose-dependant inhibition of matrix invasion, demonstrating potent antimetastatic action. Lysine, proline, ascorbic acid, manganese, and copper support collagen formation, indicating that the nutrients tested most likely promoted the stability of connective tissue.

Recent studies have documented the antiangiogenic and antimetastatic properties of the individual nutrients tested. Oak et al. (18) reported antiangiogenic and antimetastatic properties of green tea extract (GTE) by inhibition of proliferation and migration of endothelial cells and vascular smooth muscle cells and the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 as well as inhibition of the formation of new blood vessels. Our previous studies indicated that the inhibitory 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 (19).

Inhibitory effects of the nutrient combination of lysine, proline, ascorbic acid, and green tea extract on MMP-2 and MMP-9 expression and invasion

without cytotoxic effect support a potential role of these nutrients in the treatment of testicular cancer and warrant further investigation.

Acknowledgment

Dr. Rath Health Foundation, a non-profit organization, funded the research study.

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