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Original Article

Antitumor effect of ascorbic acid, lysine, proline, arginine, and green tea extract on bladder cancer cell line T-24

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Aims: Bladder cancer, the fourth highest incident cancer in men and tenth in women, is associated with a high rate of recurrence, even when treated *in situ*, and prognosis is poor once the cancer metastasizes to distant sites. Based on anticancer properties, we investigated the effect of a mixture of lysine, proline, arginine, ascorbic acid, and green tea extract on human bladder cancer cells T-24 by measuring: proliferation, matrix metalloproteinase (MMP) expression, and cancer cell invasive potential.
Methods: Human bladder cancer cells T-24 (ATCC) were grown in McCoy medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in 24-well tissue culture plates. At near confluence, the cells were treated with the nutrient mixture dissolved in media and tested at 0, 10, 50, 100, 500, and 1000 μg/mL in triplicate at each dose. Cells were also treated with PMA 200 ng/mL to study enhanced MMP-9 activity. Cell proliferation was evaluated by MTT assay, MMP activity by gelatinase zymography, and invasion through Matrigel.
Results: Nutrient mixture inhibited the T-24 cell secretion of MMP-2 and -9, with virtual total inhibition of MMP-2 at 500 μg/mL and MMP-9 at 100 μg/mL. The nutrient mixture significantly reduced the invasion of human bladder cancer cells T-24 through Matrigel in a dose-dependent fashion, with 95% inhibition at 500 μg/mL and 100% at 1000 μg/mL nutrient mixture ($P < 0.001$).
Conclusion: Our results suggest that our nutrient mixture is an excellent candidate for therapeutic use in the treatment of bladder cancer, by inhibiting critical steps in cancer development and spread, such as MMP secretion and invasion.

Key words antitumor effect, bladder cancer, MMP, nutrient mixture, T-24.

Introduction

With 63 000 new cases and over 13 000 deaths estimated in the United States in 2005, bladder cancer is the fourth leading incident cancer affecting men.[1] Current ineffective and underutilized diagnostic tools such as cytology, which does not detect low-grade tumors, and cytoscopy, an invasive procedure, generally only used when cancer is suspected, do not facilitate early detection.[2] Of newly diagnosed cases, 70–80% will present with superficial non-invasive tumors.[3] Approximately 80% of Ta and T1 tumors recur within 5 years following initial treatment.[4] Surgical removal of the tumor not only does not address metastases, but promotes such.[5] Furthermore, a recent study found that overexpression of angiopoietin-2 is highly correlated with histological stage, histological grade and poor prognosis, indicating negative survival for some patients treated by surgical resection.[6]

Standard treatment options for patients with deeply invasive tumors include radical cystectomy, external-beam irradiation, chemotherapy and combined external-beam irradiation and chemotherapy; however, these methods have not been shown to improve survival: 5-year survival occurs in 20–40% of patients where the tumors have not metastasized to distant sites and 5-year survival is rare in patients with metastasized bladder cancer.[7,8] Practitioners generally recommend entry into clinical trials and focus on palliation in the advanced stages; however, most randomized trials to date have shown only limited success in achieving longer survival, and exhibit significant toxicity to the gastrointestinal, myelosuppressive, renal and central nervous systems.[9,10]

The activity of matrix metalloproteinases (MMP) on the degradation of the extracellular matrix (ECM) plays a critical role in the formation of tumors and metastasis and has been found to correlate with the aggressiveness of tumor growth and invasiveness of the cancer.[11] Earlier work by Rath *et al*.[12] defined common pathomechanisms for all cancers, the destruction of ECM as a precondition for cancer cell invasion, growth and metastasis and suggested intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogs. Our previous studies have confirmed the concepts described by Rath and Pauling[12] and resulted in identifying a novel formulation of lysine, ascorbic acid, proline and green tea extract which has shown significant anticancer activity against a large number of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both *in vitro* [13–15] and *in vivo*.[16–18]

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In the current study, we investigated the anticancer effectiveness of this novel nutrient formulation on human bladder cancer cells *in vitro*, by measuring cell proliferation, modulation of MMP-2 and MMP-9 and invasive potential.

Materials and methods

Cancer cell lines and culture

Human bladder cancer cells T-24, obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), were grown in McCoy 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, USA). 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 (NM), dissolved in media and tested at 0, 10, 50, 100, 500, and 1000 μg/mL in triplicate at each dose. The plates were then returned to the incubator. The cells were washed with phosphate-buffered saline (PBS), and 500 μL of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT; Sigma, St Louis, MO, USA) at 0.5 mg/mL in media was added to each well. Cell proliferation was evaluated 24 h following incubation with test reagents. Culture media components were purchased from Gibco (Grand Island, NY, USA).

MTT assay

Cell proliferation was evaluated by MTT assay. The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (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 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 dimethylsulfoxide (DMSO), and absorbance was measured at 570 nm in a Bio Spec 1601 Shimadzu spectrometer (Schimadzu Scientific Instruments, Columbia, MD, USA). 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

Gelatinase zymography was used to determine MMP activity in condition media. Gelatinase zymography was performed in 10% Novex precast sodium dodecyl sulfate (SDS)–polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 μL) mixed with sample buffer was loaded and SDS–polyacrylamide gel electrophoresis was performed with Novex Tris glycine SDS buffer as described by the manufacturer (Invitrogen). 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 mmol/L Tris-HCl and 10 mmol/L $CaCl_2$ 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.

Matrigel invasion studies

Invasion studies were conducted using Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) matrix-coated 9-mm cell culture inserts (pore size 8 μm) set in 24-well plates, using a modified Boyden Chamber method as described by Albini *et al*.[19] Two hundred microliters of cell suspension (3×10^4 cells) supplemented with nutrients as specified in the design of the experiment, were seeded, in triplicate, on the insert in the well. The lower chambers also contained 5% fetal bovine serum as a chemoattractant. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 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 fixed with cold methanol and stained with hematoxylin–eosin and visually counted using an optical microscope.

Composition of nutrient mixture

Stock solution of the NM (total weight 4.2 g) prepared for testing 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 (80% polyphenol) 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Laboratory (Somerset, NJ, USA). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin-3-gallate [EGCG] 35%, and caffeine 1.0%); selenium 30 mg; copper 2 mg; manganese 1 mg.

Statistical analysis

The results were expressed as means ± SD for the groups. Data was analysed by independent sample *t*-test.

Results

Cell proliferation study

The NM showed no significant antiproliferative effect on human bladder cancer cell growth ($P = 0.435$), as shown in Figure 1.

Gelatinase zymography study

Zymography demonstrated weak bands associated with human bladder T-24 cell secretion of MMP-2 and induced expression of MMP-9 by PMA (200 ng/mL) treated cells.

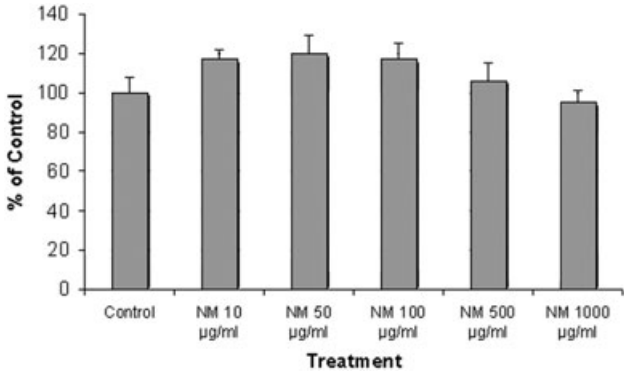

Fig. 1 Cell proliferation study: effect of a combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on a bladder cancer T-24 cell line.

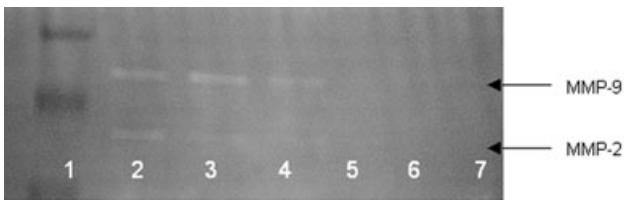

Fig. 2 The effect of the nutrient mixture (NM)of lysine, proline, arginine, ascorbic acid, and green tea extract on MMP-2 and MMP-9 secretion by bladder cancer cells was measured by gelatinase zymography in conditioned media. Lanes correspond as follows: 1, markers; 2, control; and 3–7, NM at 10, 50, 100, 500, 1000 µg/mL.

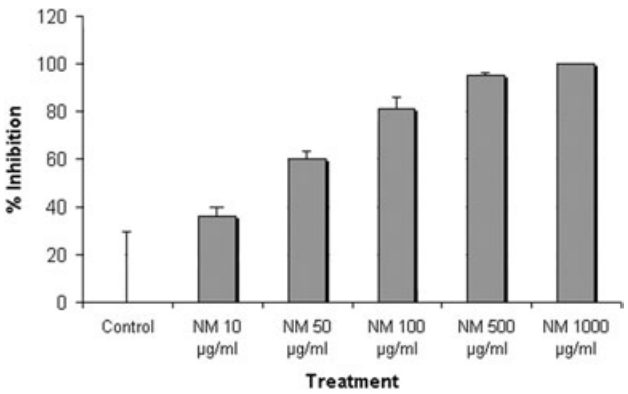

Fig. 3 Dose-dependent inhibition of T-24 Matrigel invasion by nutrient mixture with 95% inhibition at 500 µg/mL and total inhibition at 1000 µg/mL ($P < 0.001$).

The NM inhibited T-24 cell expression of MMP-2 and -9 with virtual total inhibition of MMP-2 at 100 µg/mL and MMP-9 at 500 µg/mL concentration (Fig. 2).

Invasion study

The NM significantly inhibited the invasion of human bladder cancer cells T-24 through Matrigel in a dose-dependent fashion, with 95% inhibition at 500 µg/mL and 100% at 1000 µg/mL NM ($P < 0.001$; Figs 3,4).

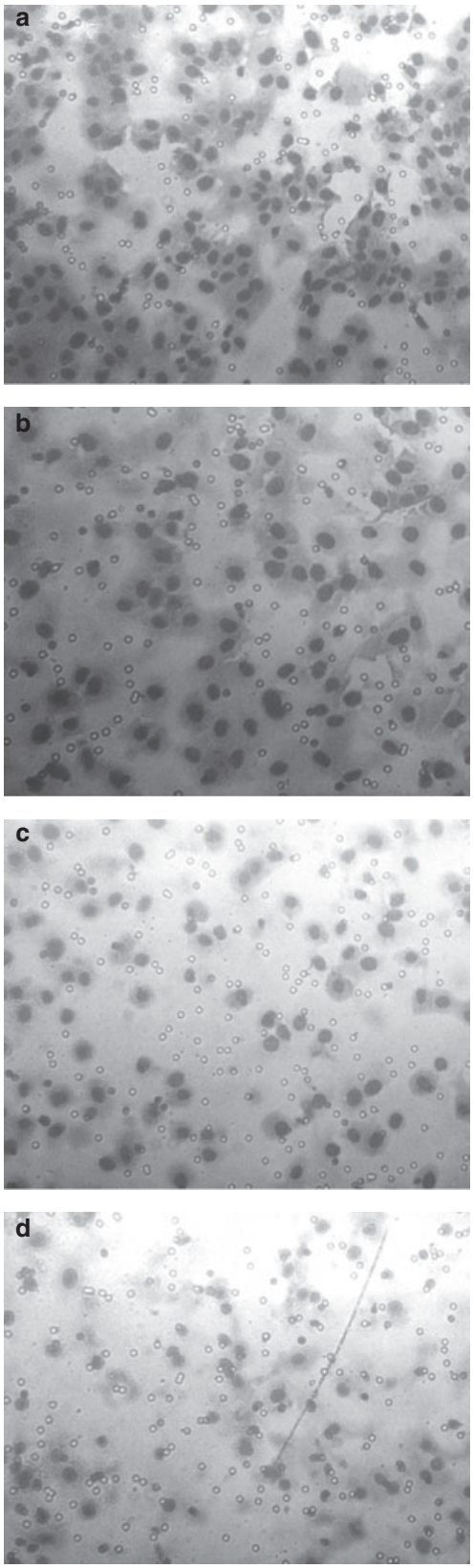

Fig. 4 Invasion photomicrographs: A, control (0 nutrient mixture [NM]); B, 50 µg/mL NM; C, 100 µg/mL NM; D, 500 µg/mL NM.

Morphology (hematoxylin–eosin staining)

Treatment with NM showed no alterations in morphology even at the highest concentration of NM.

Discussion

The results of this study exhibited complete inhibition of invasive parameters without morphological change. *In vitro* Matrigel invasion of human bladder cancer cells T-24 decreased in a dose-dependent fashion, with complete inhibition of invasion at 1000 μg/mL NM and inhibited MMP secretion with virtual total block of MMP-2 at 100 μg/mL NM and MMP-9 at 500 μg/mL NM.

One of the most promising approaches to cancer is targeting universal pathomechanisms involved in cancer growth and invasion. Cancer invasiveness can be curbed by encapsulation of tumors through a decrease in matrix degradation accompanied by optimized ECM structure and integrity. Degradation of basement membranes by MMP is key to the invasive potential of cancer cells; highly metastatic cancer cells secrete higher amounts of MMP than do poorly metastatic cells, demonstrating that the invasive and metastatic abilities of these cancer cells correlate with MMP-9 expression. Control of the proteolytic activity of the ECM provides an opportunity of addressing common mechanism of metastasis, angiogenesis and tumor growth. For example, ascorbic acid, lysine, proline, manganese, and copper have been shown to support collagen formation, and ascorbic acid and green tea extract to inhibit MMP expression of cancer cells. Rath and Pauling[12] suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogs. Lysine interferes with the activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, and consequently affecting the plasmin-induced MMP activation cascade.[12] A recent study demonstrated sevenfold reduction in metastasis of transgenic mammary cancer in plasmin-deficient mice.[20]

In addition, this mixture of nutrients probably enhanced the stability and strength of the connective tissue, as optimization of synthesis and structure of collagen fibrils depends upon hydroxylation of proline and lysine residues in collagen fibers. Ascorbic acid is essential for the hydroxylation of these amino acids and it regulates collagen synthesis at the transcriptional level. Suboptimal levels of ascorbic acid and lysine are possible in various pathological stages and in deficient diets as these nutrients are not produced in the human body.

The inhibitory effects of the individual nutrients in the mixture have been reported in both clinical and experimental studies. A recent *in vivo* study reported that green tea leaves prevented tumor initiation in rats when administered prior to injection of N-butyl-N-(hydroxybutyl)-nitrosamine-induced urinary bladder tumors.[21] Green tea extract and its derivatives have shown to exert inhibitory effects against bladder tumor growth; researchers at the Medical College of Ohio observed 100% cell lethality in AY-27 rat transitional cell cancer following exposure to EGCG, and *in vivo*, histology showed 64% tumor-free rate in Fisher 344 rats exposed to EGCG over the control.[22] Ascorbic acid has been reported to exert cytotoxic and antimetastatic actions on malignant cell lines;[23–25] in addition, low levels of ascorbic acid have been reported in cancer patients.[26–28]

Our previous studies indicated 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.[13] Furthermore, in contrast to chemotherapy, which causes indiscriminate cellular and ECM damage, morphological studies showed that even at the highest concentrations of NM, the bladder cancer cells were not affected, demonstrating that this formulation is non-toxic to cells.

By inhibition of MMP secretion and invasion our results suggest that the specific mixture of lysine, proline, ascorbic acid and green tea extract studied is an excellent candidate for preventative and therapeutic use in the treatment of bladder cancer; however, additional studies on animal models and clinical trials are necessary to more fully evaluate the role of nutrient supplementation in the treatment of bladder cancer.

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