



Anticancer effect of lysine, proline, arginine, ascorbic acid and green tea extract on human renal adenocarcinoma line 786-0

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Received May 9, 2006; Accepted July 19, 2006

Abstract. Five-year survival is limited to 60% in renal cancer patients at diagnosis. Due to the cancer's resistance to conventional treatments and associated high morbidity, we investigated the antimetastatic effects of a specific nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid and green tea extract on human renal adenocarcinoma cell line 786-0 by measuring: cell proliferation, modulation of MMP-2 and -9 secretion, and cancer cell invasive potential. Human renal cancer cell line 786-0 (ATCC) was grown in RPMI medium in 24-well tissue culture plates. At near confluence, the cells were treated with NM, dissolved in media, and tested at 0, 10, 50, 100, 500 and 1000 $\mu\text{g/ml}$ 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 secretion by gelatinase zymography, and invasion through Matrigel. Zymography demonstrated MMP-2 and MMP-9 secretion by uninduced renal cancer cells with enhanced MMP-9 induced by PMA (200 ng/ml) treatment. NM inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500- $\mu\text{g/ml}$ concentration and MMP-9 at 100 $\mu\text{g/ml}$. The invasion of renal cancer cells through Matrigel was totally inhibited ($p=0.0001$) by NM at 1000 $\mu\text{g/ml}$ concentration. Our results support a potential role for the nutrient mixture tested in the treatment of renal cell carcinoma, by inhibition of MMP-2 and MMP-9 secretion and invasion.

Introduction

The American Cancer Society estimates approximately 36,160 new cases of kidney cancer in 2005. Upon diagnosis, renal cell carcinoma is localized to the kidney in 50% of the cases, in another 25%, the cancer will have spread to nearby

tissue, and in the remaining 25% will have metastasized to distant sites; associated 5-year survival in these patient groups is roughly 90, 60 and 9%, respectively (1). Late diagnosis is largely due to diverse clinical manifestations mistaken as symptoms of non-cancerous disease; symptoms may include hematuria, fatigue, edema, fever and weight loss and generally do not present in the early stages where conventional management of the cancer is most effective (2,3).

Renal cell carcinoma (RCC) is erratic and unpredictable even when diagnosed and treated early by nephrectomy, as the neoplasm can appear to remain stable for years and then metastasize to distant locations (4). This anomaly may be epidemiological or may be due to the elimination of endostatin and angiostatin levels that prevent angiogenesis from occurring and the resulting proliferation of metastatic lesions to distant locations such as the lungs and lymph nodes (5). Traditional therapeutic approaches to RCC such as surgery, radiation therapy, chemotherapy and hormone therapy have largely been unsuccessful at controlling the progression of cancer (6-8). These methods do not address metastases and can promote tumor progression by impairing the immune system. Treatment methods focused on the regulation of tumor proliferation are necessary to effectively control RCC.

Abnormal expression of the family of zinc-dependant endopeptidases, known as matrix metalloproteinases (MMPs) can trigger degradation of the extracellular matrix (ECM), and underlying basal membranes that function as a barrier against cell migration, ultimately leading to tumoral progression. The role of MMPs on the progression of various malignancies has been reported in numerous studies (9-11). Furthermore, studies have found that MMP activity correlates with the invasiveness of the cancer.

Urokinase-type (uPA) and tissue-type (tPA) plasminogen activators that cleave plasminogen into active plasmin trigger MMP activity. In identifying the destruction of the ECM as a precondition for cancer cell invasion, growth and metastasis, Rath and Pauling proposed intervention of plasmin-induced proteolysis through such natural inhibitors, such as lysine and its analogues (12). Our previous studies have shown significant antitumoral activity of the nutrient combination (NM) tested against a number of cancer cell lines by inhibiting growth of the cancer cells, blocking tissue invasion and MMP secretion *in vitro* (13-15) and *in vivo* (16-18).

In the current study, we investigated a specific nutrient combination containing lysine, proline, arginine, ascorbic acid and green tea extract on human renal adenocarcinoma cell

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Key words: renal cancer, MMP-2, MMP-9, nutrient mixture, green tea extract, lysine, proline, arginine, ascorbic acid

line 786-0 by measuring cell proliferation, modulation of MMP-2 and -9 secretion, and cancer cell invasive potential.

Materials and methods

Cell culture. Human renal adenocarcinoma 786-0 cells [obtained from ATCC (American Type Culture Collection, Rockville, MD)] were grown in RPMI 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 NM 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 expression. The plates were then returned to the incubator.

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 µl of MTT (Sigma #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 a 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 to be 100%.

Gelatinase zymography. MMP secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel (Invitrogen Corp.) 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 CaCl₂, 5 µM ZnCl₂, 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 (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, human renal adenocarcinoma 786-0 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.

Morphology. Morphology of cells cultured for 24 h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy.

Composition of the nutrient mixture (NM). The composition of the nutrient mixture used for testing was composed of the following in the ratios indicated: vitamin C (as ascorbic acid and as Mg, Ca, and palmitoyl ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; and 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%, epigallocatechin gallate (EGCG) 35%, and caffeine 1.0%; selenium 30 µg; copper 2 mg; and manganese 1 mg.

The nutrient mixture (NM) was formulated based on targeting different physiological processes involved in cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation and its stability. In this aspect ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in collagen formation process. Collagen stability can be controlled by lysine (12) and also by N-acetyl cysteine through its inhibitory effect on MMP-9 activity (19) and invasive activities of tumor cells (20). Also, selenium has been shown to interfere with MMP expression and tumor invasion (21), as well as migration of endothelial cells through ECM (20). Ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide (22). Green tea extract has been shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression (23). Since 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 the case of breast cancer cells (24).

Based on the evidence available in literature and our own research, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetyl cysteine, selenium, copper and manganese would result from their synergy. 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 (25). 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.

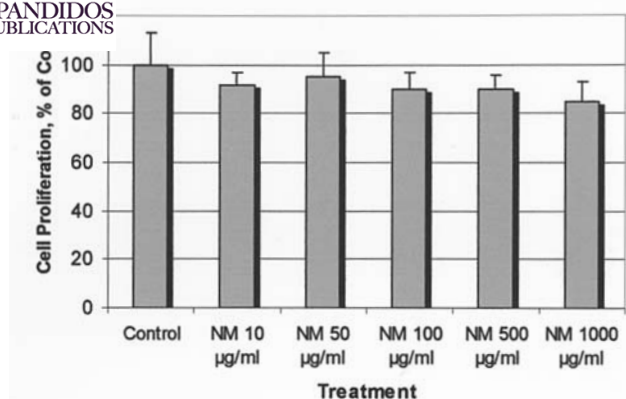


Figure 1. Effect of the nutrient mixture (NM) on growth of human renal cancer cell line 786-0: 24 h MTT assay. NM showed no significant effect on renal cancer cell growth.

Results

Cell proliferation study. The nutrient mixture (NM) showed no significant effect on renal cancer cell growth (Fig. 1).

Gelatinase zymography study. Zymography demonstrated MMP-2 and MMP-9 secretion by uninduced human renal cancer cells (Fig. 2A) with enhanced MMP-9 secretion by PMA (200 ng/ml)-treated cells (Fig. 2B). NM inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500 µg/ml concentration and MMP-9 at 100 µg/ml.

Matrigel invasion study. The invasion of human renal cancer cells through Matrigel was significantly reduced at 500 µg/ml (by 82%, $p=0.0003$) and totally inhibited ($p=0.0001$) at 1000 µg/ml concentration of NM (Fig. 3).

Morphology study (hematoxylin and eosin staining). H&E staining showed no morphological changes at the tested doses of NM (Fig. 4).

Discussion

Numerous studies have documented a correlation between increased MMP-2 and MMP-9 activity and poor survival in a

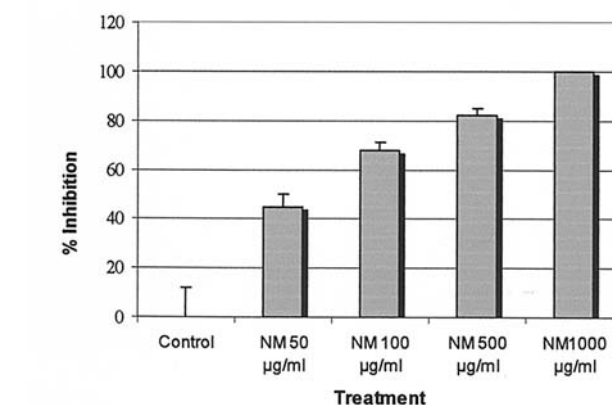


Figure 3. Effect of the nutrient mixture (NM) on human renal cancer cell 786-0 Matrigel invasion. The invasion of human renal cancer cells through Matrigel was significantly reduced at 500 µg/ml (by 82%, $p=0.0003$) and totally inhibited ($p=0.0001$) at 1000 µg/ml concentration of NM.

number of malignancies, including renal cell carcinoma (26). *In vitro*, it has been found that highly metastatic cells secrete more MMPs than do poorly metastatic cells (27). *In vivo*, studies have also documented that transgenic mice associated with overexpression of MMPs develop more cancers than do control mice (28). This data has prompted researchers to investigate 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 analogues (12). Metastatic spread to remote sites is dependant upon the ability of the tumor to penetrate the basement membrane and extracellular matrix. Matrix invasion can be prevented by strengthening the connective tissue surrounding the cancer cells, contributing to the 'encapsulation' the tumor. 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. Additionally, lysine prevents collagen-digesting enzymes from binding to plasminogen active sites, blocking the activation of plasmin by plasminogen, thereby preventing cell migration.

While the mechanistic action of the nutrients tested was not reviewed in the scope of this experiment, the dose-dependant

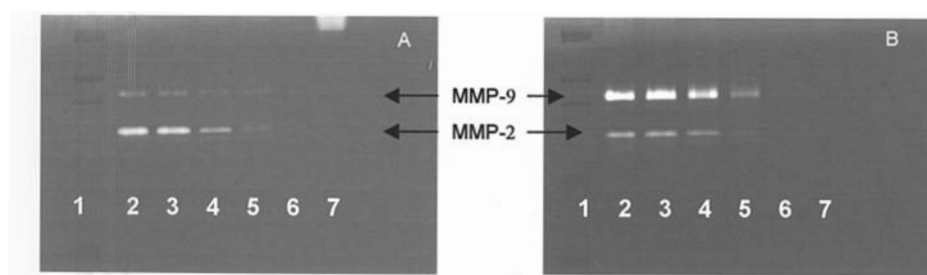


Figure 2. Effect of the nutrient mixture (NM) on MMP-2 and MMP-9 secretion by human renal cancer 786-0 cells: (A), Untreated cells; (B), PMA (200 ng/ml)-treated cells. Legend 1, markers; 2-Control, 3-7 NM 10, 50, 100, 500, 1000 µg/ml. Zymography demonstrated MMP-2 and MMP-9 secretion by uninduced human renal cancer cells and enhanced MMP-9 secretion by PMA-treated cells. NM inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500 µg/ml concentration and MMP-9 at 100 µg/ml.

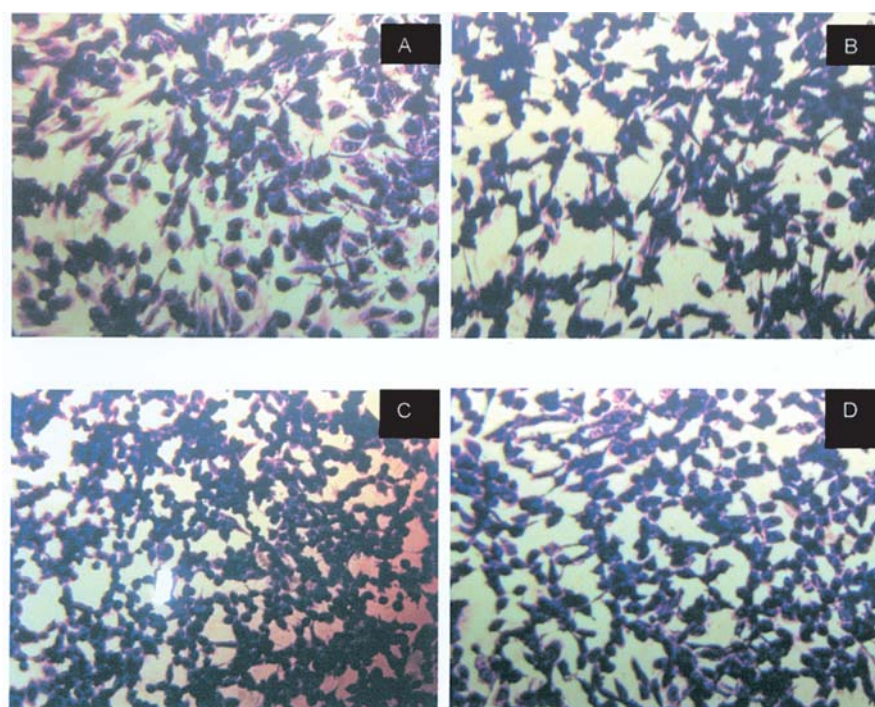


Figure 4. Effect of NM on human renal cancer cell 786-0 morphology (H&E staining): (A), Control; (B), NM 50 $\mu\text{g/ml}$; (C), 100 $\mu\text{g/ml}$; (D), 1000 $\mu\text{g/ml}$.

inhibitory effect of the nutrient mixture on MMP-2 and MMP-9 secretion by human renal adenocarcinoma cell line 786-0 was consistent with its dose-dependent inhibition of matrix invasion, suggesting that the nutrients tested enhanced the stability of the connective tissue, as lysine, proline, ascorbic acid, manganese and copper support collagen formation. Complete inhibition of invasion of renal cancer cells was seen at 1000 $\mu\text{g/ml}$ concentration NM ($p=0.0001$). The nutrient mixture inhibited MMP-2 and MMP-9 secretion in a dose-dependent fashion with virtual total inhibition of MMP-2 at 500 $\mu\text{g/ml}$ NM concentration and MMP-9 at 100 $\mu\text{g/ml}$, demonstrating potent antimetastatic action.

The inhibitory effects of the individual nutrients tested have been reported in both clinical and experimental studies. Ascorbic acid has been reported to exert cytotoxic and anti-metastatic actions on malignant cell lines (29-31); in addition, low levels of ascorbic acid have been reported in cancer patients (32-34). In another study, tumor cell invasion of a reconstituted basement membrane matrix was reduced by 50% with epigallocatechin-3-gallate (EGCG) concentrations equivalent to that in the plasma of moderate green tea drinkers, and 2 orders of magnitude below those of tissue inhibitors of MMPs, demonstrating potent antiangiogenic and antimetastatic potential (35). However, 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 (25). Furthermore, in contrast to chemotherapy, which causes indiscriminate cellular and ECM damage, morphological studies showed that even at the highest concentrations of NM, renal cancer cells were not affected, demonstrating that this formulation is non-toxic to cells.

By inhibition of MMP-2 and MMP-9 secretion and invasion, our results suggest that the mixture of lysine, proline,

ascorbic acid and green tea extract studied is an excellent candidate for preventative and therapeutic use in the treatment of renal 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 cancer.

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