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Research Article

Antitumor Effect of a Combination of Lysine, Proline, Arginine, Ascorbic Acid, and Green Tea Extract on Pancreatic Cancer Cell Line MIA PaCa-2

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Abstract

Background: Current treatment of pancreatic cancer is generally associated with poor prognosis, even if diagnosed early, owing to its aggressive rate of metastasis and non-responsiveness to chemotherapy and radiotherapy. Matrix metalloproteinases (MMPs) have received much attention in recent years for their role in various malignancies, and have been implicated in tumor invasion, metastasis, and angiogenesis.

Aim of Study: Reported antitumor properties of ascorbic acid, lysine, proline, and green tea extract prompted us to investigate the effect of a combination of lysine, proline, arginine, ascorbic acid, and green tea extract on pancreatic cancer cell line MIA PaCa-2 for viability, MMP expression, invasion, and morphology.

Methods: Viability was evaluated based on cell proliferation by MTT assay and MMP expression in condition media by gelatinase zymography. Invasion through Matrigel™ was assayed and morphology was observed by hematoxylin and eosin (H+E)staining. Data was analyzed by independent sample "*t*" test.

Results: The nutrient mixture (NM) did not inhibit cell proliferation at 10 µg/mL and exhibited a dose-dependent antiproliferative effect with maximum inhibition of 38% over the control at 1000 µg/mL. Zymography demonstrated production of only MMP-9, which showed a dose-dependent decreased expression that was abolished at 100 µg/mL of NM. Invasion through Matrigel was inhibited at 10, 50, 100, and 500 µg/mL by 66%, 66%, 87% and 100%, respectively. H&E staining did not indicate changes even at the highest concentration of NM.

Conclusion: Our results suggest that the formulation of green tea extract, lysine, proline, and ascorbic acid, tested as a promising adjunct to standard treatment of pancreatic cancer, by inhibiting MMP expression and invasion without toxic effects—important parameters in cancer metastasis.

Key Words: Pancreatic cancer; MMPs; lysine; proline; ascorbic acid; green tea extract; antitumor; MIA PaCa-2.

Introduction

Cancer of the pancreas, a highly lethal disease with the poorest likelihood of survival among all major malignancies, continues to be a major unsolved health problem, causing approx 28,000 deaths in the United States and 50,000 deaths in Europe each year. Pancreatic cancer has increased in incidence over the past few decades and ranks as the fourth leading cause of cancer-related deaths in both men and women. Despite the associated high mortality rate, the etiology of pancreatic cancer is poorly understood; the survival rate

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of exocrine pancreas is less than 4% and depends upon localization of the tumor within the capsule of the pancreas *(1–2)*. However, metastasis is associated with more than 80% of the cases.

One of the key mechanisms that cancer cells use to spread and metastasize in the body involves enzymatic destruction of the surrounding connective tissue. Therapeutic approaches for controlling this process with specific drugs have not been successful and currently there are no means available to control cancer 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, and thus facilitate cancer metastasis. There is obviously a need for safe and effective natural approaches that can be used to control the process of cancer metastasis.

All types of cancer cells form tumors and spread in the body by degrading the extracellular matrix (ECM) by means of various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. In 1992 Rath and Pauling 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 *(3)*. These nutrients can exercise their anti-tumor potential through several mechanisms, among them the inhibiting of MMPs and strengthening of connective tissue surrounding cancer cells (tumor-encapsulating effect). In a previous study, we demonstrated the anti-proliferative and anti-invasive potential of lysine, ascorbic acid, proline, and epigallocatechin gallate (EGCG) on human breast cancer (MDA-MB 231), colon cell cancer (HCT 116), and melanoma (A2058) cell lines *(4)*. The nutrient mixture (NM) containing lysine, proline, ascorbic acid, and green tea extract also suppressed the growth of these tumors, without any adverse effects, in nude mice. In the current study, we investigated the anti-tumor potential of NM in vitro on the MIA Pa Ca-2 human pancreatic cancer cell line.

Methods and Materials

Composition of the Nutrient Mixture (NM)

The stock solution of the nutrient mixture (total weight 4.4 g) used 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, 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 mg; copper, 2 mg; and manganese, 1 mg.

Cell Culture

Human pancreatic cancer cells MIA PaCa-2 were grown in DME supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) in 24-well tissue culture plates. 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, dissolved in media, and tested in triplicate at 0, 10, 50, 100, 500, and 1000 µg/mL concentrations. The plates were incubated with test reagents another 24 h and then evaluated.

MTT Assay

Viability/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 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 the absorbance was measured at 570 nm in a Bio Spec 1601, Shimadzu spectrometer. 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

MMP expression in condition 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 non-reduced conditions. Culture media (20 µL) 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 m*M* Tris-HCl and 10 m*M* $CaCl_2$ 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 mobility of known proteins.

Matrigel Invasion Studies

Invasion studies were conducted using Matrigel™ (Becton Dickinson, Franklin Lakes, NJ) 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. *(5)*, 200 µL of cell suspension (3×10^4 cells) supplemented with nutrients, as specified in the design of the experiment in triplicate, were seeded 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 remaining cells in the upper layer of the insert were carefully swabbed with cotton. The penetrating cells in the lower layer were fixed with cold methanol and stained with hematoxylin and eosin. The cells that invaded the lower side of the filter were counted using an optical microscope.

Statistical Analysis

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

Results

Pancreatic Cancer Cell Proliferation Study

The nutrient mixture did not inhibit cell proliferation at 10 µg/mL and exhibited a dose-dependent antiproliferative effect with maximum inhibition of 38% over the control at 1000 µg/mL (Fig. 1). Results were statistically significant ($p = 0.0029$).

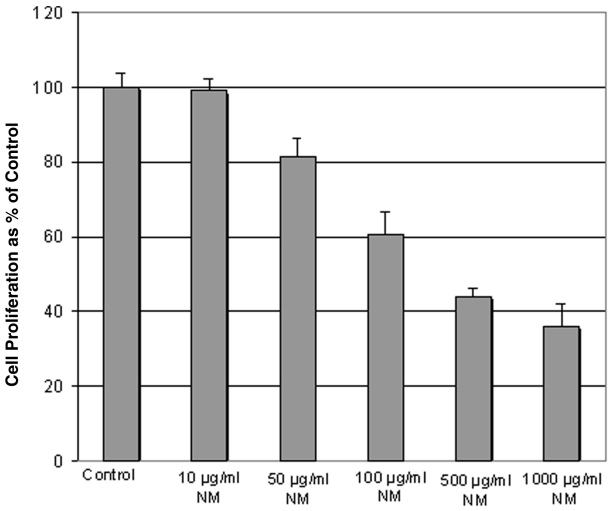

Fig. 1. Cell proliferation of human pancreatic cell line MIA PaCa-2 was measured by MTT assay 24 h after treatment with the nutrient mixture (NM) of lysine, proline, arginine, ascorbic acid, and green tea extract. Results were expressed as means ± SD for the groups. A 95% CI was used ($p = 0.0029$).

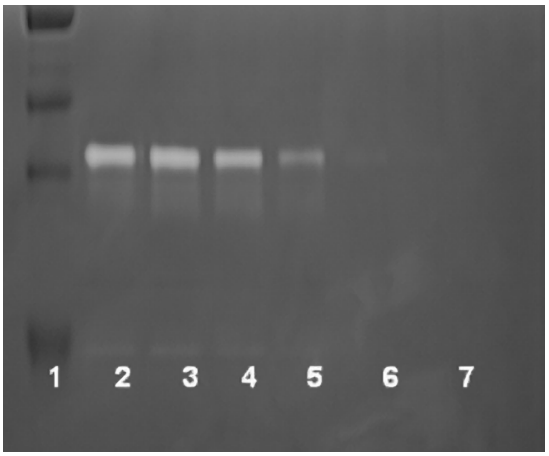

Fig. 2. The effect of the nutrient mixture (NM) on MMP-9 expression by MIA PaCa-2 cells was measured by gelatinase zymography in condition media. Lanes correspond as follows: 1, Markers; 2, Control; and 3–7, NM 10, 50, 100, 500, 1000 µg/mL.

Gelatinase Zymography Study

As shown in Fig. 2, zymography demonstrated expression of only MMP-9 by pancreatic cancer cells; MMP-9 levels showed a dose-dependent

decreased expression, which was abolished at 100 µg/mL of the nutrient mixture.

Extracellular Matrix Invasion and Migration Study

Invasion of pancreatic cancer cells through Matrigel was inhibited in the presence of 10, 50, 100, and 500 µg/mL of NM by 66%, 66%, 87%, and 100% respectively (Figs. 3A,B). H&E staining showed no morphological changes even at highest concentration of NM. Results were statistically significant ($p = 0.0077$).

Discussion

The results of this study showed that the nutrient mixture (NM) had substantial antiproliferative action (approx 60% inhibition at 500 µg/mL) without morphological changes, and complete inhibition of invasive parameters in vitro on human pancreatic cell line MIA Pa Ca-2. Matrigel invasion and MMP-9 expression of pancreatic cancer cells decreased in a dose-dependent fashion with complete inhibition of invasion and MMP expression at 500 µg/mL.

Our main objective in this study was to test the effect of NM on multiple parameters of cancer growth and invasion. 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. Matrix invasion can be affected with inhibition of MMP expression as well as by increasing stability and strength of the connective tissue surrounding the cells, contributing to the "encapsulation" of the tumor. The dose-dependent inhibitory effect of the nutrient combination of lysine, proline, arginine, ascorbic acid, and green tea extract on MMP-9 expression of the pancreatic cancer cells was consistent with its dose-dependent inhibition of matrix invasion. 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 on hydroxylation of proline and lysine residues in collagen fibers; ascorbic acid is essential for the hydroxylation of these amino acids.

The inhibitory effects of lysine, proline, ascorbic acid, and green tea extract (EGCG) have been reported in clinical as well as experimental studies. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines; in addition, low levels of ascorbic acid have been reported in cancer patients *(6–11)*. EGCG is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines *(12–14)*.

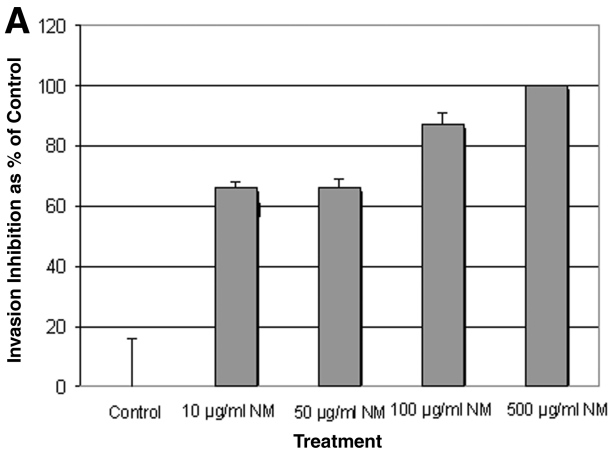

Fig. 3. **(A)** Invasion studies on the effect of the nutrient mixture (NM) on MIA Pa Ca-2 cell invasion through Matrigel™. Results were expressed as means ± SD for the groups. NM inhibited invasion by 87% at 100 µg/mL and by 100% at 500 µg/mL ($p = 0.0077$). **(B)** *(opposite page)* The cells that had penetrated the Matrigel membrane and migrated into the lower chamber were stained with Hematoxylin and Eosin and visually counted under the microscope.

However, individual nutrients are not as powerful as nutrient synergy. Our previous studies demonstrated 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 *(4)*. Furthermore, in contrast to chemotherapy which causes indiscriminate cellular and ECM damage, morphological studies of pancreatic cancer cells treated with NM showed that even at the highest concentrations of NM, the pancreatic cells were not adversely affected, demonstrating that this formulation is safe for cells.

Conclusions

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer

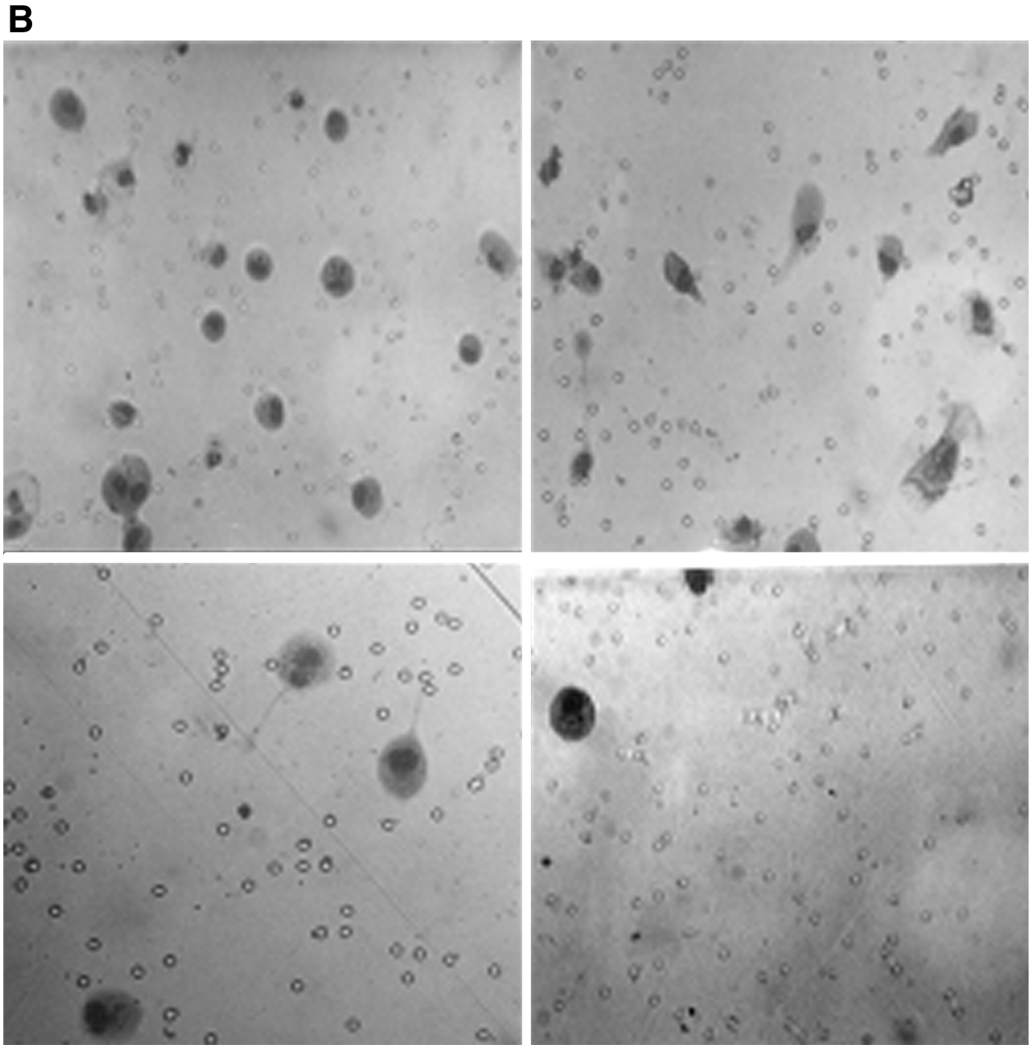

Fig. 3. **(B)** *(Continued from previous page.)*

prevention and treatment, the results of this study suggest the formulation of lysine, proline, arginine, ascorbic acid, and green tea extract, tested as a valuable and promising candidate for therapeutic use in the treatment of pancreatic cancer, by inhibiting cell proliferation, MMP expression, and invasion. The effective concentrations of the nutrient mixture (50–200 µg/mL) in our study correspond to the reference range of amino acid plasma values for healthy human individuals *(14)*. Amino acid plasma in this range responds positively to oral supplementation *(15)*. Epigallocatechin gallate (EGCG) plasma concentration has been shown to reach 3 µ*M* secondary to supplementation with a single oral dose in healthy volunteers *(16)*, which corresponds to approx 100 µg/mL of the nutrient mixture used in our study. Thus, we conclude that the biological effects described in this study are relevant to physiological conditions

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