Suppression of human cervical cancer cell lines Hela and DoTc2 4510 by a mixture of lysine, proline, ascorbic acid, and green tea extract

M.W. ROOMI, V. IVANOV, T. KALINOVSKY, A. NIEDZWIECKI & M. RATH
Matthias Rath Research Institute, Cancer Division, Santa Clara, California


Cervical cancer, the second most common cancer in women, once metastasized, leads to poor prognosis. We investigated the antitumor effect of a nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid, and green tea extract on human cervical cancer cells Hela (CCL-2) and DoTc2 4510 by measuring cell proliferation (MTT assay), modulation of matrix metalloproteinases (MMP)-2 and MMP-9) expression (gelatinase zymography), and cancer cell invasive potential (Matrigel). NM showed significant antiproliferative effect on CCL-2 and DoTc2 4510 cancer cells. The NM inhibited CCL-2 expression of MMP-2 and MMP-9 in a dose-dependent fashion, with virtual total inhibition of MMP-2 at 1000 µg/mL and MMP-9 at 500 µg/mL. Untreated DoTc2 4510 cells showed MMP-9 expression, which was enhanced with phorbol 12-myristate 13-acetate treatment. NM inhibited MMP-9 expression in a dose-dependent fashion, with virtual inhibition at 500 µg/mL. Invasion of human cervical cancer cells CCL-2 and DoTc2 4510 through Matrigel decreased in a dose-dependent fashion, with 100% inhibition at 500 µg/mL NM (p < 0.0001) and 1000 µg/mL NM (p < 0.0001), respectively. Our results suggest that the mixture of lysine, proline, arginine, ascorbic acid, and green tea extract has potential in the treatment of cervical cancer by inhibiting critical steps in cancer development and spread.

Keywords: antitumor effect, cervical cancer, DOTC2 4510, Hela, MMP, nutrient mixture.

Cervical cancer is the second leading cancer affecting women, with approximately 10,370 new cases and over 3700 deaths estimated in the United States for 2005, despite the availability and reliability of the Pap smear. While patient survival is favorable in early-stage cervical cancer, patient outcome greatly suffers once the cancer has metastasized to distant sites. The standards of treatment include radiation therapy, chemotherapy, and surgery. External radiotherapy involves the indiscriminate attack of all cells, causing cellular damage and destruction of the body’s connective tissue, and facilitating cancer metastasis. Once cervical cancer metastasizes, 5-year survival is limited to 20–30%. In addition, studies have demonstrated that the primary tumor inhibits angiogenesis in its distant metastasis and successful surgical removal of the tumor can trigger metastasis. Such data indicate that new, more effective treatment methods are necessary.

Cancer cells secrete proteases that can, directly or indirectly (through the activation of other proteases), degrade the extracellular matrix (ECM). Proteolysis is the result of the activation of proteases on other proteases in a cascade: aspartyl protease activates cysteine proteases that can activate pro-uPA, which can convert plasminogen to plasmin. Cysteine protease and plasmin can activate zymogens of matrix metalloproteinases (MMPs). MMPs, especially MMP-2 and MMP-9, lead to ECM degradation, resulting in cancer progression. The activity of MMPs has also been found to correlate with the aggressiveness of tumor growth and invasiveness of the cancer.

In defining common pathomechanisms for all cancers, the destruction of ECM as a precondition for
cancer cell invasion, growth, and metastasis, Rath and Pauling proposed intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogues. Previous studies have shown significant anticancer activity of the nutrient combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (EGCG) against a number of cancer cell lines by blocking cancer growth, tissue invasion, and MMP expression both in vitro and in vivo.

In the current study, we investigated the antitumor effect of nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid, and green tea extract on human cervical cancer cells in vitro by measuring cell proliferation, modulation of MMP-2 and MMP-9 expression, and invasive potential.

Materials and methods

Cell culture

Human cervical Hela (CCL-2) and carcinoma DoTaC2 4510 (CRL 7920) cells, obtained from ATCC (American Type Culture Collection, Rockville, MD), were grown in Dulbecco's Modified Eagle's (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 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 and 500 μL of MTT (Sigma #M-2128) 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 obtained from Gibco (Grand Island, NY).

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 [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 dimethyl sulfoxide (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 expression in conditioned media was determined by gelatin zymography. Gelatinase zymography was performed in 10% Novex precast sodium dodecyl sulfate (SDS)–polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 μL) mixed with loading buffer were loaded, and SDS–polyacrylamide gel was performed with Tris–glycine SDS buffer, as described by the manufacturer (Novex, San Diego, CA). 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.

Matrigel invasion studies

Invasion studies were conducted using Matrigel (Becton Dickinson, Franklin Lakes, NJ) inserts in 24-well plates. Suspended in medium, human cervical cancer Hela and carcinoma DoTaC2 4510 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 (H&E) and visually counted under the 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, and standardized green tea extract 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Lab, Somerset, NJ). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, caffeine 1.0%, selenium 30 mg, copper 2 mg, and manganese 1 mg.

Statistical analysis
The results were expressed as means ± SD for the groups. Data were analyzed by independent sample t test.

Results

Cell proliferation study
NM with and without phorbol 12-myristate 13-acetate (PMA) 200 ng/mL showed significant antiproliferative effect on human cervical cancer Hela cell growth (untreated 37%, treated 57% at 1000 μg/mL)[P < 0.0002], as shown in Figure 1A. B. NM significantly inhibited cervical DoTc2 4510 cancer cell growth (untreated 45%, treated 49%; P < 0.0004), as shown in Figure 1C, D.

Gelatinase zymography study
Zymography showed expression of MMP-2 by untreated cervical Hela and enhanced MMP-2 expression and induced MMP-9 expression by PMA (200 ng/mL)-treated Hela cells. NM inhibited the Hela expression of MMP-2 and MMP-9 in a dose-dependent fashion, with virtual total inhibition of MMP-2 at 1000 μg/mL and MMP-9 at 500 μg/mL concentration (Fig. 2A, B). Untreated DoTc2 4510 cells showed MMP-9 expression, which was enhanced with PMA treatment. NM inhibited MMP-9 expression in a dose-dependent fashion, with virtual inhibition at 500 μg/mL (Fig. 2C, D).

Invasion study
The NM significantly reduced the invasion of human cervical cancer Hela cells through Matrigel in a
dose-dependent fashion, with 76% inhibition at 100 μg/mL and 100% at 500 μg/mL NM ($P < 0.0001$; Fig. 3A). Invasion inhibition of cervical cancer DoT2 4510 cells reached 97% at 500 μg/mL NM and 100% at 1000 μg/mL NM ($P < 0.0001$; Fig. 3B).

**Morphology (H&E)**

Even at the highest concentration of NM, morphology of human cervical cancer Hela and DoT2 4510 cells was not affected (Fig. 4A–J).

**Discussion**

Matrix invasion can be controlled 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-2 and MMP-9 expression of the cervical cancer cells Hela and DoT2 was consistent with its dose-dependent inhibition of matrix invasion. Additionally, the mixture of nutrients most likely 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 and ascorbic acid is essential for the hydroxylation of these amino acids.

It is now well accepted that MMPs promote the progression of cancer by degradation of ECM components consisting of the basement membranes and interstitial connective tissue. Of all MMPs, MMP-2 and MMP-9 are considered to cause the highest level of collagen IV degradation, the main component of basement membranes and therefore play a critical role in tumoral progression and invasion. Thus, new treatment approaches are targeting universal pathomechanisms involved in cancer growth and invasion. Cancer invasiveness can be impeded through the encapsulation of tumors by a decrease in matrix degradation accompanied by optimized ECM structure and its integrity.

Studies have demonstrated that the invasive and metastatic abilities of cancer cells correlate with MMP expression as highly metastatic cancer cells secrete higher amounts of MMPs than do poorly metastatic cells\textsuperscript{15-18}. Thus, treatment methods aimed at the control of proteolytic activity of ECM allow the opportunity to focus on common mechanisms of metastasis, angiogenesis, and tumor growth. Ascorbic acid, lysine, proline, manganese, and copper are correlated with the support of collagen formation\textsuperscript{8}, and ascorbic acid\textsuperscript{19} and green tea extract\textsuperscript{20} have been linked to the inhibition of MMP expression of cancer cells. Rath and Pauling\textsuperscript{8} suggested targeting plasmin-mediated mechanisms

Figure 4. Morphology (H&E). A) Hela, control, B) Hela-NM 50 μg/mL, C) Hela-NM 100 μg/mL, D) Hela-NM 500 μg/mL, E) Hela-NM 1000 μg/mL, F) Do7c2 4510-Control, G) Do7c2 4510-NM 50 μg/mL, H) Do7c2 4510-NM 100 μg/mL, I) Do7c2 4510-NM 500 μg/mL, and J) Do7c2 4510-NM 1000 μg/mL.
with the use of nutritional components, such as lysine and lysine analogues. Lysine prevents the activation of plasminogen into plasmin by tissue plasminogen activator by binding to plasminogen active sites and consequently thwarts the plasmin-induced MMP activation cascade\(^8\). A sevenfold reduction in metastasis of transgenic mammary cancer in plasmin-deficient mice was observed in a recent study\(^21\).

In this study, complete inhibition of invasion of human cervical cancer cells Hela through Matrigel was achieved at 500 \(\mu\)g/mL concentration NM, and in cervical cancer cells DoTe2 4510, 97% inhibition was seen at 500 \(\mu\)g/mL concentration NM \((P < 0.0001)\), showing drastic antiinvasive effects. The inhibitory effects of the individual nutrients tested have been reported in both clinical and experimental studies. The clinical efficacy of EGCG was undertaken in a randomized controlled clinical trial on patients with human papillomavirus–infected cervical lesions\(^22\). Results showed a 69% overall response rate (35/51) in the treated group as compared with a 10% response rate (4/39) in untreated controls \((P < 0.05)\). In another study, cell growth rate was examined after treatment for 4, 7, and 10 days with 0–100 \(\mu\)M EGCG in primary human endocervical cells and ectocervical cell, and 90% growth inhibition was observed\(^23\). Ascorbic acid has been reported to exert cytotoxic and antimitastatic actions on malignant cell lines\(^24\)–\(^26\), in addition, low levels of ascorbic acid have been reported in cancer patients\(^27\)–\(^29\).

Our previous studies indicated that the synergistic anticancer effect of ascorbic acid, proline, lysine, and green tea extract on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients\(^9\). Furthermore, in contrast to chemotherapy, which causes indiscriminate cellular and ECM damage, morphologic studies showed that even at the highest concentrations of NM, the cervical cancer cells were not affected, showing that this formulation is nontoxic to cells.

By inhibition of MMP-2 and MMP-9 expression and invasion, our results suggest that the mixture of lysine, proline, ascorbic acid, and green tea extract is an excellent candidate for preventative and therapeutic use in the treatment of cervical 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.

References
Antitumor effect of nutrients on cervical cancer cells


