

Synergistic Effect of Combination of Lysine, Proline, Arginine, Ascorbic Acid, and Epigallocatechin Gallate on Colon Cancer Cell Line HCT 116

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ABSTRACT

Limitation of current treatment methodologies to control metastasis, as well as the proposed antitumor properties of specific nutrients, prompted us to investigate the effect of a specific formulation (NS) of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on human colon cancer cells HCT 116 for viability, MMP expression, invasion, and morphology. Cell proliferation was based on MTT assay and MMP expression in condition media by gelatinase zymography. Invasion through Matrigel™ was evaluated and morphology was assessed by Hematoxylin and Eosin staining. NS did not demonstrate antiproliferative effect up to 1000 mcg/ml concentration. Cancer cell invasion through Matrigel was significantly reduced at 100 mcg/ml (76%, $p=0.0008$) and completely inhibited at 500 mcg/ml ($p=0.0002$). Gelatinase zymography showed dose-dependent inhibition of MMP-9 expression by NS with virtual total inhibition at 100 mcg/ml. Our results suggest that the specific formulation of nutrients tested is an excellent candidate for therapeutic use in the treatment of colon cancer by inhibiting MMP expression and invasion.

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INTRODUCTION

Colorectal cancer is the second most deadly cancer in the United States; the American Cancer Society estimates that approximately 56,000 Americans will die from the disease this year. Colon cancer affects both men and women over age 50 with approximately the same frequency. Other than age, several other risk factors are associated with colorectal cancer: family/individual history of colorectal cancer or adenomatous polyps, inflammatory bowel disease, diets rich in saturated fat, and inactivity.¹ However, it is important to note that 75% of colorectal cancer cases occur in individuals with no known predisposing factors.²

While colorectal cancer is treatable upon early detection, once the cancer metastasizes to the lymph, liver, or other areas, 5-year survival is less than 10%; most of these fatalities are associated with metastasis.³ Improved screening methods have been linked to the recent decline in incidence and mortality of colorectal cancer; however, they are underutilized on basis of cost, discomfort, inaccuracy, and risk of complication, approximately 2 perforations for every 1,000 colonoscopies performed.⁴

Early-stage colon cancer is generally successfully treated with surgery (local excision/colon resection) depending on the size of the lesion; however, side effects can range from mild to severe: diarrhea, constipation,

depression, bleeding, and infection, and 15% of all colorectal patients require a permanent colostomy.⁵ Standard treatment of Stage II colon cancer and advanced stages may involve both chemotherapy and radiation therapy. As with most chemotherapy approaches, cancer cells are eventually capable of independent growth, invasion, adhesion, angiogenesis, and avoidance of apoptosis, rendering this approach ineffectual.⁶ Furthermore, only 10–20% of patients on fluorouracil experience palliation, yet the associated side effects of chemotherapy include nausea, vomiting, hair loss, mouth sores, diarrhea, fatigue, bleeding, infection, and weight loss.⁵ Finally, radiotherapy may be used before surgery to shrink tumors or postoperatively to eradicate remaining cancer cells. External radiotherapy focuses on cancer cell destruction, but not metastases, which are the main cause of death in patients with colorectal cancer.⁷ These treatment methods have not only been ineffective in providing a cure, but involve the indiscriminate attack of all cells, causing cellular damage and destruction of the body's connective tissue, facilitating cancer metastasis. Clearly, there is a need for safe and effective therapeutic approaches that can be used to control the process of cancer metastasis as well as to prevent of colon cancer.

Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through 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.⁸ These nutrients can exercise their anti-tumor effect through inhibition 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.⁹ NS also suppressed the growth of these tumors, with no adverse effects, in nude mice. In the current study, we investigated the anti-tumor potential of NS on human colon cancer cells HCT 116 by measuring: cytotoxicity, secretion of MMP-9, and matrix invasive potential.

MATERIALS AND METHODS

Cell Culture

Human colon cancer cells HCT 116 were obtained from ATCC (American Type Culture Collection, Rockville, MD) and cultured in MEM (modified Eagle's medium), supplemented with 10% fetal bovine serum, penicillin G sodium (100 mcg/ml), streptomycin (100 mg/ml), and amphotericin (0.25 mcg/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 NS (composed of vitamin C 700 mg, L-lysine 1000 mg, L-proline 750 mg, L-arginine 500 mg, N-acetyl-cysteine 200 mg, standardized green tea extract (82% polyphenol, 36% EGCG) 1000 mg, selenium 30 mg, copper 2 mg, and manganese 1 mg), dissolved in media, and tested in triplicate at 0, 10, 50, 100, 500, and 1000 mcg/ml concentrations. The plates were then returned to the incubator. Cell proliferation was evaluated after 24 hrs following incubation with test reagents. Culture media components were purchased from Gibco (Grand Island, New York), and all other chemicals used were purchased from Sigma (St. Louis, MO).

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 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 hours, 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 of DMSO (dimethyl sulfoxide), and absorbance was measured at 570 nm in a BioSpec 1601 Shimadzu spectrophotometer. The OD₅₇₀ of the DMSO solution in each well was considered proportional to the number of cells. The OD₅₇₀ 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 Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin. Culture media (20 µl) was loaded and SDS-PAGE was performed with a tris-glycine SDS buffer, as described by the manufacturer (Novex). Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 minutes 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 Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes 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) inserts in 24-well plates. Suspended in medium,

colon 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 hours. 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.

Statistical Analysis

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

RESULTS

Colon Cancer Cytotoxicity/Proliferation Study

NS was not toxic to colon cancer cells (HCT 116) even at 1000 μ g/ml concentration (Figure 1).

Gelatinase Zymography Study

As shown in Figure 2, zymography demonstrated expression of MMP-9 by human colon cancer cells HCT 116; NS inhibited MMP-9 expression in a dose-dependent fashion with virtual total inhibition at 500 μ g/ml concentration.

Invasion Study

Invasion of colon cancer cells (HCT 116) through Matrigel was significantly (76%, $p=0.008$) reduced by NS at 100 mg/ml and completely (100%, $p=0.0002$) inhibited at 500 mg/ml of NS, respectively (Figure 3A). Morphology was observed using Hematoxylin and Eosin staining. H&E stains did not show any alterations in morphology with different doses of NS (Figure 3B).

Figure 1. Antiproliferative effect of NS on colon cancer cells HCT 116 was measured by MTT assay 24 hours after treatment of NS. Results were expressed as means \pm SD for the groups.

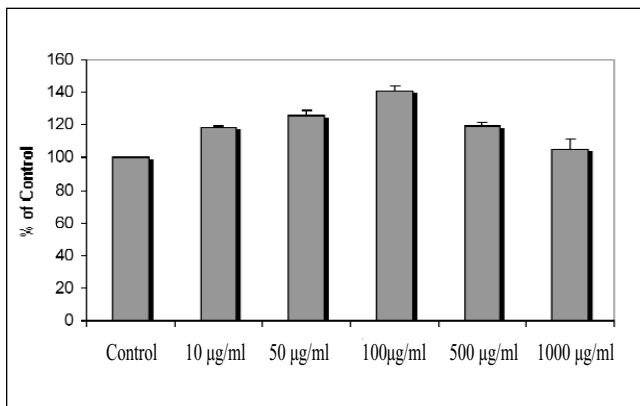


Figure 2. Effect of NS on MMP-9 expression by HCT 116 cells was measured by gelatinase zymography in condition media. Lanes correspond as follows: 1-Markers, 2-Control, and 3-7 NS 10, 50, 100, 500, 1000 μ g/ml.

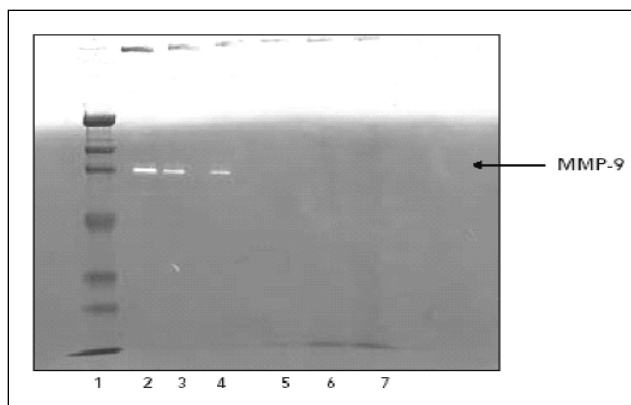
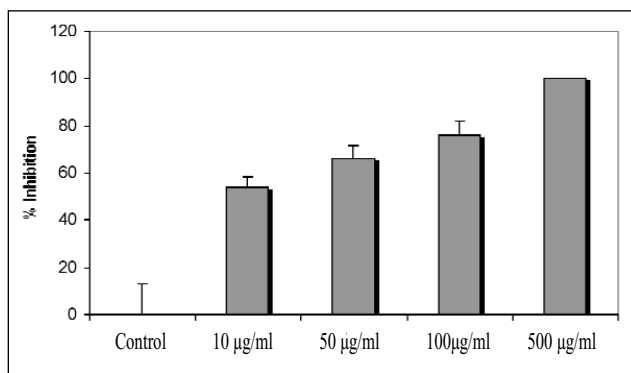


Figure 3. Invasion studies on the effect of NS on colon cancer HCT 116 cell line. Results were expressed as means \pm SD for the groups. NS significantly inhibited invasion by 76% ($p=0.0008$) at 100 μ g/ml and completely inhibited invasion (100%, $p=0.0002$) at 500 μ g/ml.



DISCUSSION

Degradation of basement membranes by MMPs is key to the invasive potential of cancer cells. Moreover, research has shown that highly metastatic colon cells (LuM1) secrete higher amounts of MMP-9 than do poorly metastatic cells, demonstrating that the level of tumoral invasion correlates with MMP-9 expression in colon cancer.¹⁰ While the results of this study showed that NS did not have any cytotoxic effect on the tested colon cancer cell line (HCT 116) even at high concentrations, NS did show substantial inhibition of invasion and MMP-9 expression at 100 μ g/ml, clearly demonstrating antimetastatic ability.

Matrix invasion can be controlled by inhibition of MMP expression, as well as by enhancing connective tissue strength and stability. In this study, the dose-dependent inhibitory effect of NS on MMP-9 expression of the colon cancer cells was consistent with its dose-dependent inhibition of matrix invasion. It has been postulated that lysine can act as a natural inhibitor of collagen matrix degradation

through its inhibitory effect on plasmin.⁸ In addition, lysine can compete for enzyme binding sites in MMPs, thereby controlling activation pathways for various types of MMPs. The mechanistic aspects of MMP-9 inhibition by lysine were not in the scope of this study.

In addition, matrix invasion by cancer cells can be modulated by increased stability and strength of the connective tissue, secondary to the activity of the nutrients provided in NS. Optimization of synthesis and structure of collagen fibrils depends upon hydroxylation of hydroxyproline and hydroxylysine residues in collagen fibers. It is well known that ascorbic acid is essential for the hydroxylation of these amino acids, but as it is not produced in the human body, sub-optimal levels are common. Additionally, low levels of ascorbic acid have been reported in cancer patients.¹¹⁻¹³

The inhibitory effects of the individual nutrients composing NS have been reported in both clinical and experimental studies. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines.¹⁴⁻¹⁶ In a six-week in vivo experiment, supplementation of EGCG caused a 60% reduction in colonic preneoplastic tumors, suggesting potent anti-inflammatory and chemopreventative properties.¹⁷

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.⁹ In contrast to radiotherapy and chemotherapy, which cause indiscriminate cellular and ECM damage, morphological studies showed that even at the highest concentrations of NS, the colon cancer cells were unaffected, demonstrating that this formulation is safe to cells.

CONCLUSION

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of this study suggest the formulation of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate tested as a valuable and promising candidate for therapeutic use in the treatment of colon cancer, by inhibiting cell proliferation, MMP expression, and invasion.

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