Anti-angiogenic effects of a nutrient mixture on human umbilical vein endothelial cells

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Abstract. Matrix metalloproteinases (MMPs) have been recognized as key players in the degradation of the extracellular matrix (ECM) by migration and proliferation of endothelial cells and their subsequent invasion of the underlying stroma. The prevention of ECM degradation through the inhibition of MMP activity has been shown to be a promising therapeutic approach to block the invasion that occurs during angiogenesis. In previous studies, we demonstrated the anti-tumor effect of a nutrient mixture (NM) containing ascorbic acid, lysine, proline, green tea extract, arginine, N-acetyl cysteine, selenium, copper and manganese on various tumor cell lines *in vivo* and *in vitro*. The aim of the present study was to determine whether this mixture has anti-angiogenic effects on human umbilical vein endothelial cells (HUVECs). At near confluence, the HUVEC cell cultures were tested with NM at 0, 10, 50, 100, 500, and 1000 μg/ml in triplicate at each dose for proliferation, migration, MMP expression, and invasion. Cell proliferation was evaluated by MTT assay, invasion potential by Matrigel invasion, MMP expression by gelatinase zymography, and cell migration by a 2 mm-wide scratch in plates. For tube formation, HUVECs were cultured in previously polymerized Matrigel. NM inhibited HUVEC migration, MMP expression and invasion through Matrigel in a dose-dependent manner. Zymography showed a dose-dependent inhibition of MMP-2 expression with virtual total inhibition at a 500 μg/ml concentration. Invasion through Matrigel was totally inhibited at 500 μg/ml NM. NM reduced cell migration by scratch test in a dose-dependent fashion with total inhibition at a 500 μg/ml concentration. NM also inhibited the tube formation of HUVECs, but did not significantly inhibit cell proliferation. These results together with our earlier findings suggest that NM is a relatively non-toxic formulation with anti-angiogenic effects, such as inhibiting vascular tube formation and endothelial cell invasion and migration.

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Introduction

Angiogenesis, the formation of new capillaries from existing blood vessels, is associated with progressive growth and metastasis of solid tumors, which depend on the recruitment of new blood vessels. The regulation of angiogenesis is achieved through a balance of pro- and anti-angiogenic stimuli. The angiogenic process is controlled by two major pro-angiogenic factors, matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) and vascular endothelial growth factor (VEGF) that acts in concert to promote angiogenesis. Blood vessels local to the tumor respond to the elaboration of VEGF and fibroblast growth factor (FGF) in malignant cells, inducing local blood vessels to sprout branches to feed the metastases. This causes micrometastases (<2 mm in size and functionally dormant) to grow beyond the 2-mm size and become a threat to the patient (1).

VEGF is specific and critical for blood vessel formation and one of the most powerful stimulators of angiogenesis. VEGF is secreted by tumor cells and promotes the proliferation of endothelial cells and remodeling of neovessels. Since endothelial cells can communicate directly with tumor cells by producing growth-promoting factors, the interrelationship between endothelial and tumor cells, and the imbalance between angiogenic factors, can promote tissue neovascularization. Angiogenic inhibitors that target both the proliferating endothelial and tumor cell compartments may therefore lead to therapeutic regulation of tumor growth (2).

Earlier work by Rath and Pauling 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 analogues (3). A critical step in angiogenesis is the degradation of ECM by the migration of endothelial cells and their subsequent invasion of the underlying stroma of neighboring tissues, where they organize into new capillary structures. This process requires the production of matrix-degrading enzymes by the invading endothelial cells.

The prevention of ECM degradation through the inhibition of MMP activity, particularly MMP-2 (gelatinase A) and MMP-9 (gelatinase B), has been shown to be a promising therapeutic approach to blocking the invasion process that occurs during angiogenesis and tumor progression. The identification of novel angiogenic inhibitors that target both proliferating endothelial and tumor cells and MMP inhibitors

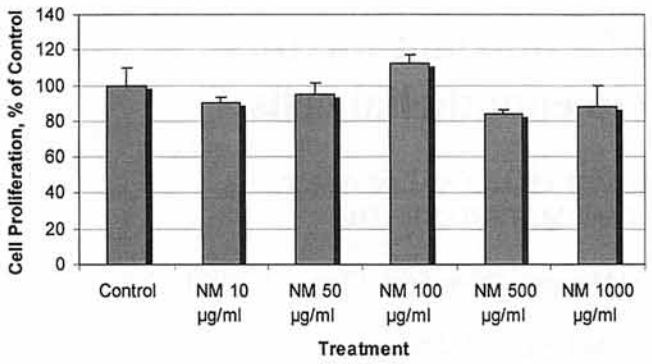

Figure 1. Effect of the nutrient mixture (NM) on HUVEC proliferation (MTT assay).

may therefore lead to the therapeutic regulation of tumor growth. Recently, several MMP inhibitors and anti-angiogenic agents have been developed. An increasing number of clinical trials are testing the therapeutic efficacy and tolerance of angiogenic agents, targeting MMPs, angiogenic growth factors, and their receptors (4).

In previous studies, we demonstrated the anti-tumor effect of a mixture containing lysine, proline, ascorbic acid and green tea extract (nutrient mixture; NM) on various human cancer cell lines *in vivo* and *in vitro* (5-11). Since angiogenesis depends on the interaction between tumor cells and endothelial cells, the aim of the present study was to determine whether NM also exhibits anti-angiogenic effects on endothelial cells.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) obtained from the American Type Culture Collection (Rockville, MD) were grown in M199 media supplemented with 10% FBS, 50 μg/ml endothelial cell growth supplement, 100 μg/ml heparin and 20 mM Hepes 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% 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.

MTT assay. The effects of NM on HUVEC proliferation were 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. After the 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, formazan product was dissolved in 1 ml DMSO, and 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 conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex 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. 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 $CaCl_2$, and 5 μM $ZnCl_2$, 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.

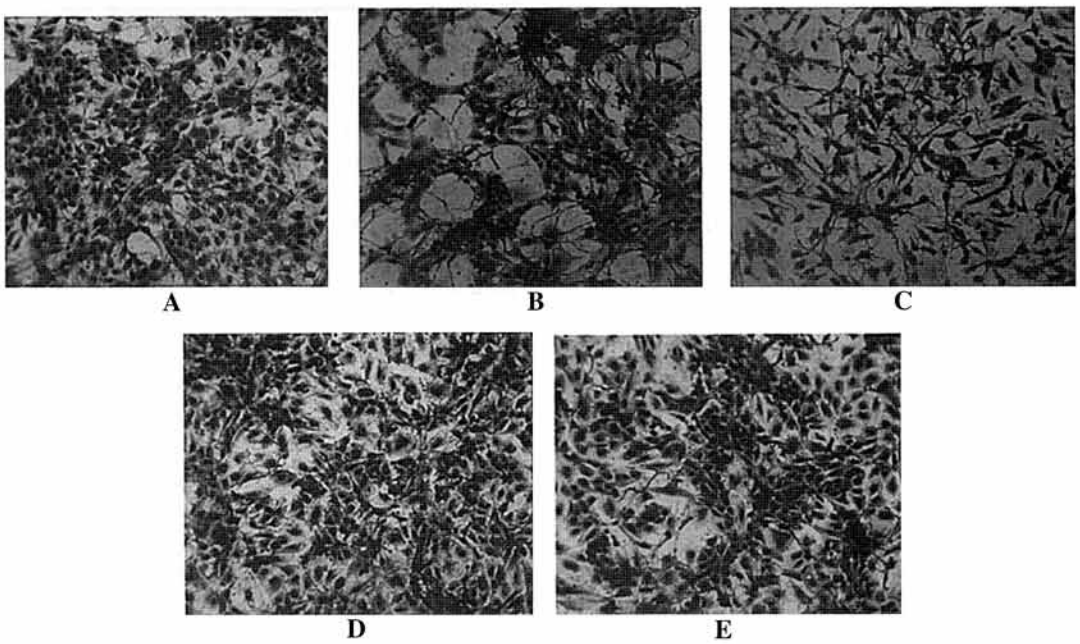

Figure 2. Effect of the nutrient mixture (NM) on HUVEC morphology. (A) Untreated control, (B) 50 μg/ml NM, (C) 100 μg/ml NM, (D) 500 μg/ml NM, and (E) 1000 μg/ml NM.

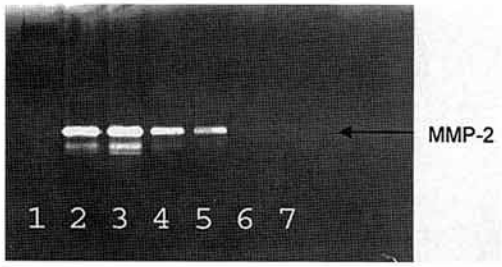

Figure 3. Effect of the nutrient mixture (NM) on HUVEC MMP-2 expression. Lane 1, markers; lane 2, control; lanes 3-7, 10, 50, 100, 500, and 1000 μg/ml NM, respectively.

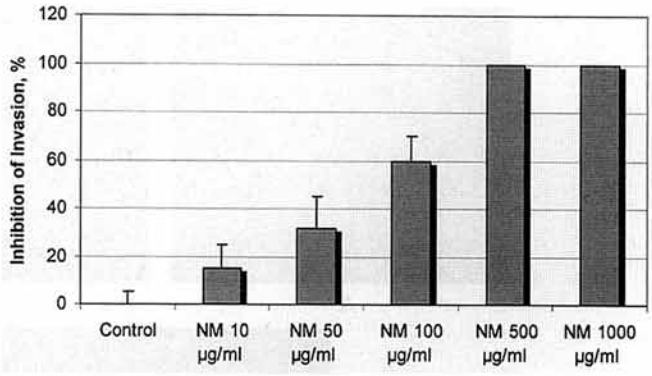

Figure 4. Effect of the nutrient mixture (NM) on HUVEC Matrigel invasion and migration.

Matrigel invasion studies. Invasion studies were conducted using Matrigel™ (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, HUVECs 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% CO_2 for 24 h. After incubation, the media from the wells were withdrawn. Cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. 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.

HUVEC capillary tube formation on Matrigel study. Unpolymerized Matrigel was placed in wells (300 μl/well) of a 24-well tissue culture plate and allowed to polymerize for 1 h at 37°C. HUVEC cells were plated in triplicate at a density of 100,000 cells per well with NM at 0, 10, 50, 100, 500, and 1000 μg/ml concentrations. After 24 h incubation in 5% CO_2 humidified atmosphere at 37°C, endothelial cells were washed with PBS, fixed and stained with H&E, tube morphology was observed and photomicrographs were taken.

HUVEC migration assay. At near confluence, a 2-mm wide single uninterrupted scratch from top to bottom of the culture plates was made. Culture plates were washed with PBS and incubated with NM in medium and tested at 0, 10, 50, 100, 500, and 1000 μg/ml in triplicate at each dose for 24 h. The cells were washed with PBS, fixed and stained with H&E. Photomicrographs at a x100 magnification were taken.

Composition of the nutrient mixture (NM). Stock solution of the nutrient mixture (total weight 4.2 Gm) is composed of: 700 mg vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate); 1000 mg L-lysine; 750 mg L-proline; 500 mg L-arginine; 200 mg N-acetyl cysteine; and 1000 mg standardized green tea extract (green tea extract derived from green tea leaves) obtained from U.S. Pharma Lab. The certificate of analysis indicates the characteristics: 80% total polyphenol, 60% catechins, 35% EGCG, and 1.0% caffeine (80% polyphenol); 30 mg selenium; 2 mg copper; and 1 mg manganese.

We formulated and tested NM since multiple effects of cancer inhibition were being observed at different stages of cancer growth and metastasis. For example, the ECM integrity is dependent on adequate collagen formation; the amino acids lysine and proline are necessary for the formation of collagen chains, and ascorbic acid is essential for the hydroxylation reaction. Manganese and copper are also essential for collagen formation. Ascorbic acid has also been shown to inhibit cell division and growth through production of hydrogen peroxide (12). Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer (13). N-acetyl cysteine has been observed to inhibit MMP-9 activity (14) and invasive activities of tumor cells (15), as well as endothelial tissue invasion (4). Selenium has been shown to interfere with MMP expression and tumor invasion (16), and migration of endothelial cells through ECM (4,15). 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 in breast cancer cells (17).

Based on the evidence available in literature and our own research, we hypothesized that a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetyl cysteine, selenium, copper and manganese would work synergistically. 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 (18). 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 a 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 using independent sample t-tests.

Results

Effect of NM on HUVEC proliferation (MTT assay). The nutrient mixture had no significant effect on HUVEC proliferation (Fig. 1). HUVEC morphology was unchanged even at the highest concentration (Fig. 2).

Effect of NM on HUVEC MMP expression (gelatinase zymography). As shown in Fig. 3, zymography demonstrated expression of MMP-2 by human umbilical vein endothelial cells (HUVECs). NM inhibited the expression of MMP-2 in

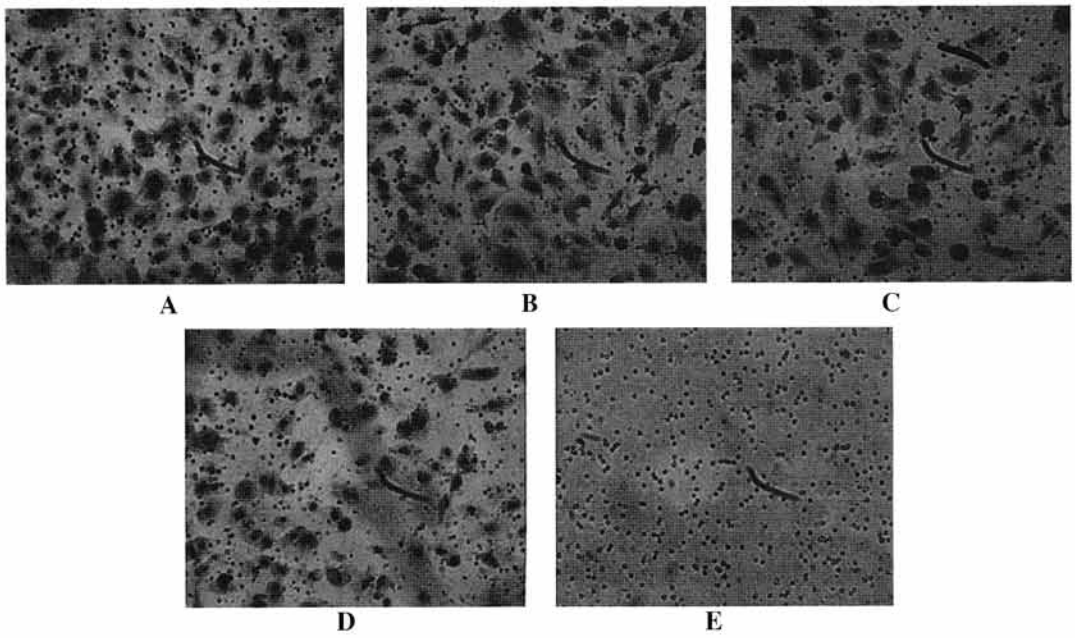

Figure 5. HUVEC invasion photomicrographs. (A) Control, (B) 10 μg/ml NM, (C) 50 μg/ml NM, (D) 100 μg/ml NM, and (E) 500 μg/ml NM.

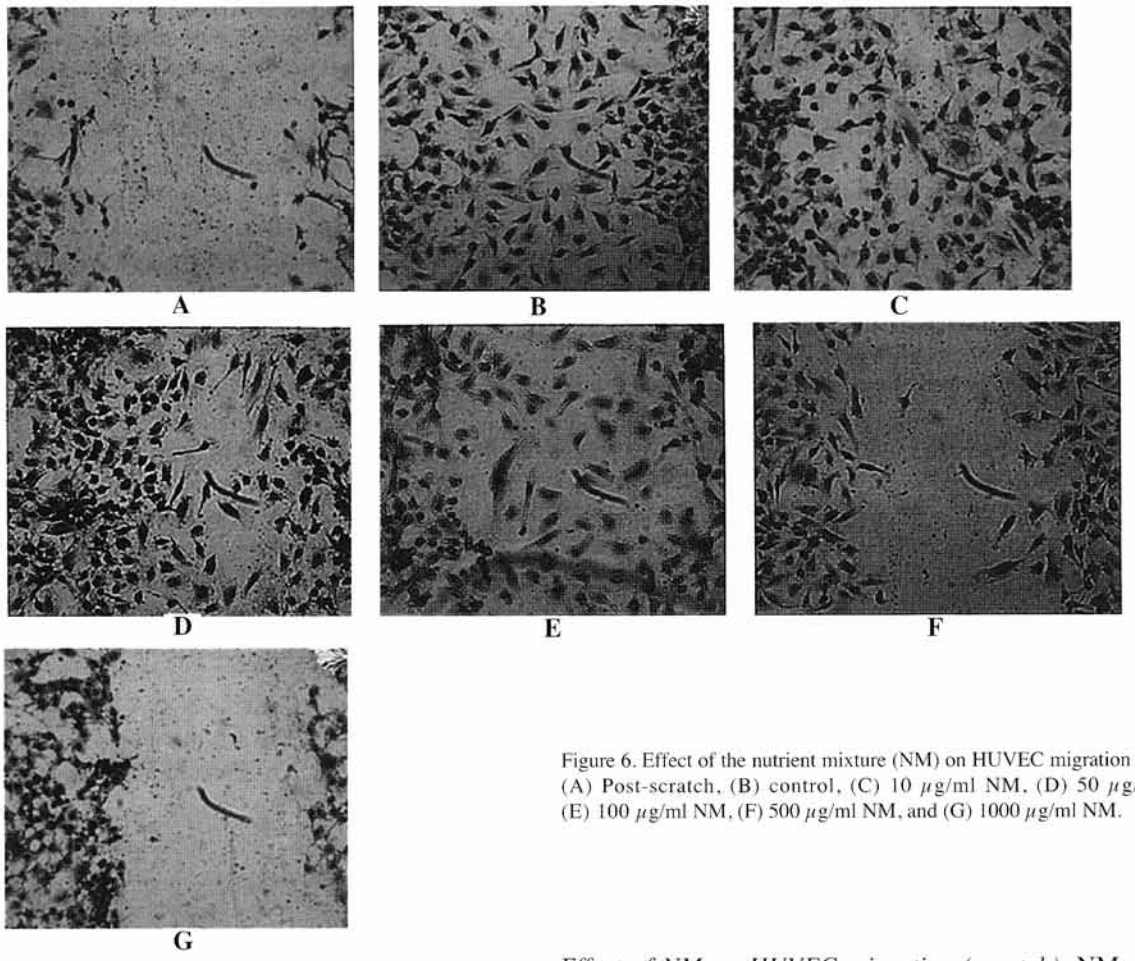

Figure 6. Effect of the nutrient mixture (NM) on HUVEC migration (scratch). (A) Post-scratch, (B) control, (C) 10 μg/ml NM, (D) 50 μg/ml NM, (E) 100 μg/ml NM, (F) 500 μg/ml NM, and (G) 1000 μg/ml NM.

a dose-dependent fashion with virtual total inhibition at a 500 μg/ml concentration.

Effect of NM on HUVEC invasion (Matrigel). Invasion of HUVECs through Matrigel was reduced by 60% at 100 μg/ml and totally inhibited at 500 μg/ml ($p<0.0001$) (Figs. 4 and 5).

Effect of NM on HUVEC migration (scratch). NM reduced HUVEC migration by scratch test in a dose-dependent fashion with total inhibition at a 500 μg/ml concentration, as shown in Fig. 6A-G.

Effect of NM on HUVEC capillary tube formation assay. The inhibitory effect of NM on capillary tube formation (*in vitro* differentiation) was assessed by exposure to NM of HUVECs

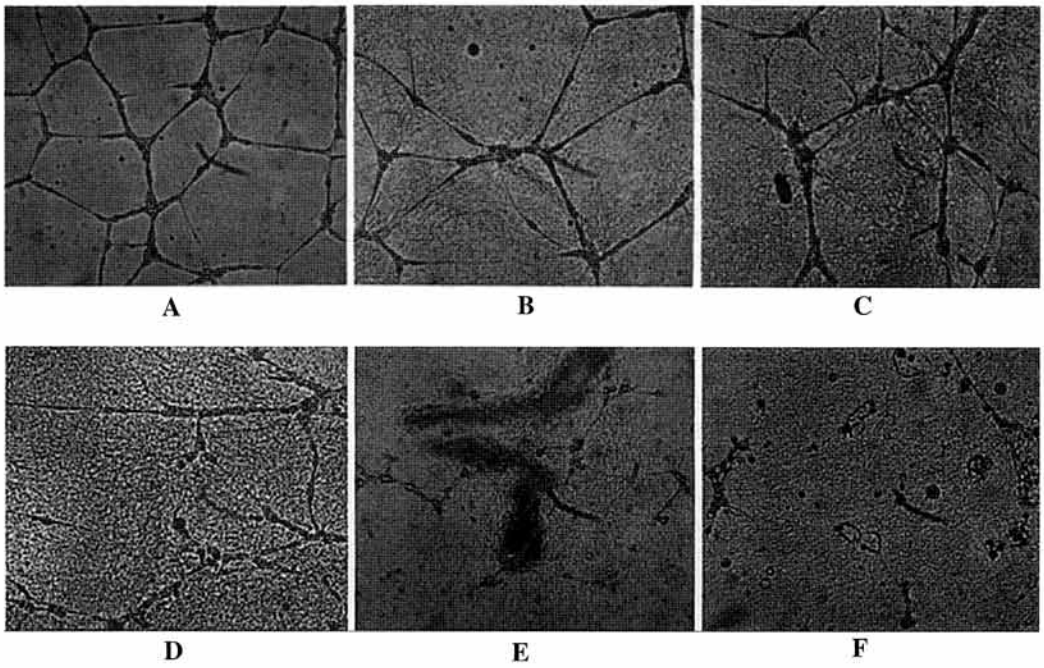

Figure 7. Effect of the nutrient mixture (NM) on HUVEC capillary tube formation assay (H&E, original magnification, x100). (A) Control, (B) NM 10 μg/ml, (C) NM 50 μg/ml, (D) NM 100 μg/ml, (E) NM 500 μg/ml, and (F) NM 1000 μg/ml.

on Matrigel. After 22 h, the control showed that HUVEC formed hollow tubes on Matrigel in contrast to NM-treated HUVECs, which demonstrated a dose-dependent inhibition of capillary tube formation with complete disruption of capillary tubes at 1000 μg/ml NM (Fig. 7A-F; H&E stain, original magnification, x100).

Discussion

Angiogenesis and vascular remodeling play significant roles in tumor growth. Endothelial cell activation is the first process to occur under pathological and physiological conditions. Cytokines from various sources are released in response to hypoxia or ischemia and activated endothelial cells penetrate new areas of the body by degradation of the basement membrane by MMPs. In a previous study, we found that the nutrient combination of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine and tea phenolics significantly inhibited cytokine expression by human aortic smooth muscle cells induced by TNF-α and LPS (19).

In this study, we showed that NM inhibited HUVEC Matrigel invasion and expression of MMP-2 in a dose-dependent fashion with virtual total inhibition at a 500 μg/ml concentration, implying inhibition of angiogenesis by blockage of ECM degradation and invasion by endothelial cells. In addition, NM significantly inhibited HUVEC *in vitro* migration (scratch test).

These results were consistent with the effect of the nutrient mixture *in vitro* on HUVEC capillary tube formation. Significant inhibition of HUVEC vascular network formation on Matrigel beds was seen at the low concentration of 50 μg/ml NM. Inhibition of tube formation increased with increasing NM dose, resulting in complete disruption at 500 μg/ml NM. This ability to disrupt the integrity of preformed tubes indicates that NM may not only prevent, but also regress new blood vessels. Capillary tube formation in the basement membrane-like matrix of Matrigel requires that endothelial cells adhere to and move on the extracellular matrix, a process dependent mainly on various integrins (20). Capillary tube formation is a complex process requiring cell-matrix interactions, intercellular communications, as well as cell motility. The inhibitory effect of NM on HUVEC vascular network formation in light of other *in vitro* studies indicates that NM inhibits attachment, migration and invasion of endothelial cells.

Oncologists throughout North America, Europe, Asia and Australia have developed and tested more than 60 anti-angiogenic and MMP inhibitor drugs for patients with a wide variety of solid tumors. Unfortunately, many compounds have limited efficacy, due to problems of delivery and penetration and moderate effects on the tumor cells, accompanied by severe toxicity and damage to healthy tissues. In addition, the activity of these compounds is mainly limited by the development of drug resistance (21).

In this and previous studies (5-11), the relatively non-toxic nutrient mixture (NM) has shown potent anti-tumor and anti-angiogenic activity *in vivo* and *in vitro*. While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of these studies suggest that this formulation is an excellent candidate for adjunctive therapeutic use in the treatment of metastatic cancer by inhibiting endothelial MMP expression, invasion and migration without toxic effects.

References

1. Wahl MR, Moser TL and Pizzo SV: Angiostatin and anti-angiogenic therapy in human disease. Recent Prog Horm Res 59: 73-104, 2004.
2. Cross MJ and Claesson-Welsh L: FGF and VEGF function in angiogenesis: signaling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci 22: 201-207, 2001.
3. Rath M and Pauling L: Plasmin-induced proteolysis and the role of apoprotein(a), lysine and synthetic analogs. Orthomol Med 7: 17-23, 1992.

4. Tossetti F, Ferrari N, De Flora S and Albini A: Angioprevention: angiogenesis is a common and key target for cancer chemopreventative agent. FASEB J 16: 2-14, 2002.
5. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: Antitumor effect of nutrient synergy on human osteosarcoma cells U-2OS, MNNG-HOS and Ewing's sarcoma SK-ES.1. Oncol Rep 13: 253-257, 2005.
6. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: In vitro and in vivo antitumorigenic activity of a mixture of lysine, proline, ascorbic acid and green tea extract on human breast cancer lines MDA MB-231 and MCF-7. Med Oncol 22: 129-138, 2005.
7. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: Synergistic effect of combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on colon cancer cell line HCT 116. JANA 7: 40-43, 2004.
8. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: Antitumor effect of a combination of lysine, proline, arginine, ascorbic acid, and green tea extract on pancreatic cancer cell line MIA PaCa-2. Int J Gastrointest Cancer 35: 97-102, 2005.
9. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: *In vivo* antitumor effect of ascorbic acid, lysine, proline and green tea extract on human colon cancer cell HCT 116 xenografts in nude mice: evaluation of tumor growth and immunohistochemistry. Oncol Rep 13: 421-425, 2005.
10. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: *In vivo* antitumor effect of ascorbic acid, lysine, proline and green tea extract on human prostate cancer PC-3 xenografts in nude mice: evaluation of tumor growth and immunohistochemistry. In Vivo 19: 179-183, 2005.
11. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: Modulation of N-methyl-N-nitrosourea induced mammary tumors in Sprague-Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract. Breast Cancer Res 7: R291-R295, 2005.
12. Maramag C, Menon M, Balaji KC, *et al*: Effect of vitamin C on prostate cancer cells *in vitro*: effect of cell number, viability and DNA synthesis. Prostate 32: 188-195, 1997.
13. Hare Y: Green tea: health benefits and applications. Marcel Dekker, Basel, New York, 2001.
14. Kawakami S, Kageyama Y, Fujii Y, *et al*: Inhibitory effects of N-acetyl cysteine on invasion and MMP 9 production of T24 human bladder cancer cells. Anticancer Res 21: 213-219, 2001.
15. Morini M, Cai T, Aluigi MG, Noonan DM, *et al*: The role of N-acetyl cysteine in the prevention of tumor invasion and angiogenesis. Int J Biol Markers 14: 268-271, 1999.
16. Yoon SO, Kim MM and Chung AS: Inhibitory effects of selenite on invasion of HT 1080 tumor cells. J Biol Chem 276: 20085-20092, 2001.
17. Cooke JP and Dzau VJ: Nitric oxide synthase: Role in the genesis of vascular disease. Annu Rev Med 48: 489-509, 1997.
18. Roomi MW, Ivanov V, Niedzwiecki A and Rath M: Synergistic antitumor effect of ascorbic acid, lysine, proline, and epigallocatechin gallate on human fibrosarcoma cells HT-1080. Ann Cancer Res Ther 12: 6-11, 2004.
19. Ivanov V, Ivanova S, Roomi MW, Kalinovsky T, Niedzwiecki A and Rath M: A nutrient mixture containing ascorbic acid, lysine, proline, arginine, cysteine, and green tea extract suppresses autocrine inflammatory response in cultured human aortic smooth muscle cells. Res Commun Pharmaco Toxicol 9: 3-16, 2004.
20. Friedlander M, Brooks PC, Schaffer RW, Kincaid CM, Varner JA and Cheresh DA: Definition of two angiogenic pathways by distinct α integrins. Science 270: 1500-1502, 1995.
21. Griffioen AW and Molema G: Angiogenesis: potential for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharm Rev 52: 237-268, 2000.