

Anti-Angiogenic Functional and Medicinal Foods

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26 A Novel Nutrient Mixture Containing Ascorbic Acid, Lysine, Proline, and Green Tea Extract Inhibits Critical Parameters in Angiogenesis

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CONTENTS

26.1	Introduction	562
26.2	Composition of the Nutrient Mixture	563
26.3	The Effect of NM on Surrogate Models for Angiogenesis	565
26.3.1	CAM Study	565
26.3.2	In Vivo Mouse Matrigel Plug Assay	565
26.4	The Effect of NM on Human Osteosarcoma MNNG-HOS Cells In Vivo	566
26.5	The Effect of NM on In Vitro Studies in Human Osteosarcoma U2OS Cells	567
26.6	Effect of NM on Human Osteosarcoma	568
26.6.1	U2OS MMP Activity: Gelatinase Zymography	568
26.7	Effect of NM on Human Osteosarcoma U2OS Cell Matrigel Invasion.....	570
26.8	Effect of NM on Human Osteosarcoma U2OS Secretion of VEGF, IL-6, IL-8, FGF, and TGFb	570
26.9	Effect of the Nutrient Mixture on Endothelial Cells	571
26.10	Effect of NM on HUVEC Capillary Tube Formation	571
26.11	Effect of NM on HUVEC Proliferation: MTT Assay 24 h	572
26.12	Effect of NM on HUVEC Morphology (H&E)	573
26.13	Effect of NM on HUVEC MMP Expression: Gelatinase Zymography	573
26.14	Effect of NM on HUVEC Matrigel Invasion	575
26.15	Effect of NM on HUVEC Migration	576
26.16	Discussion	576
	References	577

26.1 INTRODUCTION

Angiogenesis, the formation of new capillaries from existing blood vessels, is essential for progressive growth, invasion, and metastasis of solid tumors. Over 2500 scientific reports demonstrate the dependency of tumor growth on angiogenesis [1]. Angiogenesis not only allows the tumor to increase in size, but it also provides a route for metastasis to distal sites in the body. The degree of vascularization in a tumor has been correlated with the metastatic potential and prognosis of the disease [2].

The regulation of angiogenesis is achieved through a balance of pro- and anti-angiogenic stimuli. Two major factors driving angiogenesis are matrix metalloproteinases (MMPs) that degrade ECM, and vascular endothelial growth factor (VEGF), a stimulatory factor for cell migration. The prevention of ECM degradation through the inhibition of MMP activity, in particular 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. Vascular endothelial growth factor is specific and critical for blood vessel formation, and is one of the most powerful stimulators of angiogenesis.

Blood vessels local to the tumor respond to the malignant cells' elaboration of VEGF and fibroblast growth factor (FGF), inducing local blood vessels to sprout branches to feed the metastases. This causes small micrometastases to grow beyond the 2-mm size, which is functionally dormant, and become a threat to the patient when rapid growth causes local damage [3]. Vascular endothelial growth factor is secreted by tumor cells and promotes the proliferation of endothelial cells by binding to cell surface receptors, as well as the migration toward the tumor. 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 and angiogenic inhibitors can promote tumor vascularization. Other stimulating factors include angiopoietin-1, epidermal growth factor (EGF), tumor necrosis factor alpha (TNF- α), interleukin (IL)-1, IL-6, IL-8, and platelet derived growth factor (PDGF).

Physiologically, angiogenesis is suppressed by one or more of the known endogenous inhibitors, such as angiostatin, endostatin, thrombospondin, and tissue inhibitors of metalloproteinases. Angiostatin, a fragment of plasminogen endogenously produced by tumors, is found naturally in significant amounts in the circulation of patients with primary tumors; angiostatin levels can control metastatic cell proliferation until a primary tumor is removed [4,5]. However, when primary tumors are surgically removed, the endogenous levels of angiostatin and other inhibitors decrease and micrometastases that have previously seeded elsewhere in the body are allowed to grow. Angiostatin, however, has a short half-life of the peptide, requiring continuous administration. Efforts have been made to identify other anti-angiogenic agents as potential cancer treatments.

Earlier work by Rath and Pauling [6] defined common pathomechanisms for all cancers, the destruction of ECM as a precondition for cancer cell invasion, metastasis, and angiogenesis, and suggested intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogues. The prevention of

ECM degradation through the inhibition of MMP activity, in particular 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 may, therefore, lead to the therapeutic regulation of tumor growth. Most angiogenic inhibitors also act as anti-invasive or antimetastatic agents. 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 [7].

Our previous work confirmed the direction described by Rath and Pauling [6] and resulted in identifying a novel formulation of lysine, proline, ascorbic acid, and EGCG-enriched green tea extract (NM) that has shown significant anti-cancer activity against a large number of cancer cell lines—blocking cancer cells' growth, tissue invasion and MMPs' activity both in vitro and in vivo [8–14]. The aim of this study was to determine the effectiveness of this novel nutrient formulation as an inhibitor of angiogenesis using both in vitro and in vivo models.

26.2 COMPOSITION OF THE NUTRIENT MIXTURE

The nutrient mixture (NM) is composed of the following relative amounts of components: 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 U.S. Pharma Lab). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, and caffeine 1.0% (80% polyphenol), selenium 30 µg, copper 2 mg, and manganese 1 mg.

We formulated and tested NM because we were looking at the multiple effects of cancer inhibition at different stages of cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation; the amino acids lysine and proline are necessary for 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 [15]. Green tea extract has been shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer [16]. N-acetyl cysteine has been observed to inhibit MMP-9 activity [17] and the invasive activities of tumor cells [18], as well as endothelial tissue invasion [7]. Selenium has been shown to interfere with MMP expression and tumor invasion [19], as well as the migration of endothelial cells through ECM [7,18]. 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 breast cancer cells [20].

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 $\mu\text{g/ml}$ EGCG to that of 50 $\mu\text{g/ml}$ [21]. 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.

The presence of an adequate blood supply is required for the growth and metastasis of malignant tumors; thus, inhibition of tumor-induced angiogenesis represents a promising approach for cancer therapy. A number of *in vivo* and *in vitro* models have been developed facilitating the study of angiogenesis. Using various *in vivo* and *in vitro* models, we demonstrated that the nutrient mixture of lysine, proline, ascorbic acid, and green tea extract had anti-angiogenic properties.

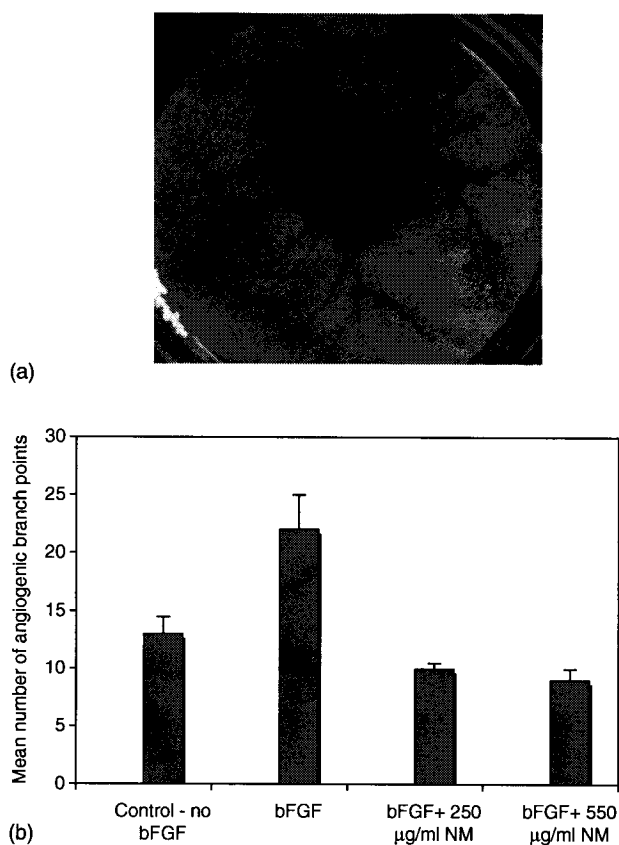


FIGURE 26.1 Effect of NM on bFGF-induced angiogenesis in chick CAM assay.

26.3 THE EFFECT OF NM ON SURROGATE MODELS FOR ANGIOGENESIS

Angiogenesis induces a release of various angiogenic factors, among them bFGF. We applied the chick embryo chorioallantoic membrane (CAM) assay to test the effects of NM, as this is a comprehensive *in vitro* system in tissue that incorporates all angiogenic processes in one mode. This assay utilizes a microenvironment in which angiogenesis naturally occurs and provides a good model for evaluation of systemically administered antagonists. In addition, it allows selection of inhibitors of angiogenesis that interfere with new blood vessel development without affecting pre-existing vessels.

26.3.1 CAM STUDY

The chick CAM angiogenesis assay was performed essentially as described by Brooks, et al. [22]. Briefly, the CAMs of 10-day old chick embryos were separated from the shell membrane. Filter discs previously coated with cortisone acetate were saturated with 15 μ l of recombinant bFGF at a concentration of 1.0 μ g/ml. The embryos were allowed to incubate for a total of 24 h. The embryos were next treated with a single I.V. injection of NM (250 or 500 μ g/embryo) in a total volume of 100 μ l. At the end of a 3-day incubation period, the embryos were sacrificed and the CAMs were resected and washed. The number of branching angiogenic blood vessels were counted within the confines of the filter discs for each CAM for each experimental condition. The nutrient mixture caused a significant ($P < 0.50$) reduction (from 22 to 10 blood vessel branch points within the confined region of the filter disc) in bFGF-induced angiogenesis as compared to no treatment (bFGF only) (Figure 26.1) [23]. The number of blood vessel branch points is relative to the number of newly sprouting angiogenic vessels.

26.3.2 IN VIVO MOUSE MATRIGEL PLUG ASSAY

The anti-angiogenic effects of NM observed in the CAM study were congruent with our *in vivo* mouse Matrigel study, which showed that NM, included as a component of a diet, strongly suppressed bFGF-induced angiogenesis in C57BL/6J female mice. To investigate the anti-angiogenic potential of NM, an extract of basement membrane proteins (Matrigel) impregnated with bFGF, an inducer of neovascularization, was injected subcutaneously into C57BL/6J female mice; Passaniti [24] found that a subcutaneous injection of Matrigel, supplemented with angiogenic factors, into C57BL/6J mice reconstituted into a gel and supported an intense vascular response.

The mouse Matrigel plug assay was performed as described by Passaniti, et al. [24]. Nutrient mixture 5 mg/ml and bFGF 400 ng/ml in PBS were mixed with Matrigel in proportions not exceeding 1% of the total volume of Matrigel. A mixture of 0.5 ml Matrigel with bFGF with NM was injected s.c into four C57BL/6J female mice and the mixture of 0.5 ml Matrigel with bFGF in vehicle were injected s.c. into another group of four C57BL/6J female mice. After seven days, mice were

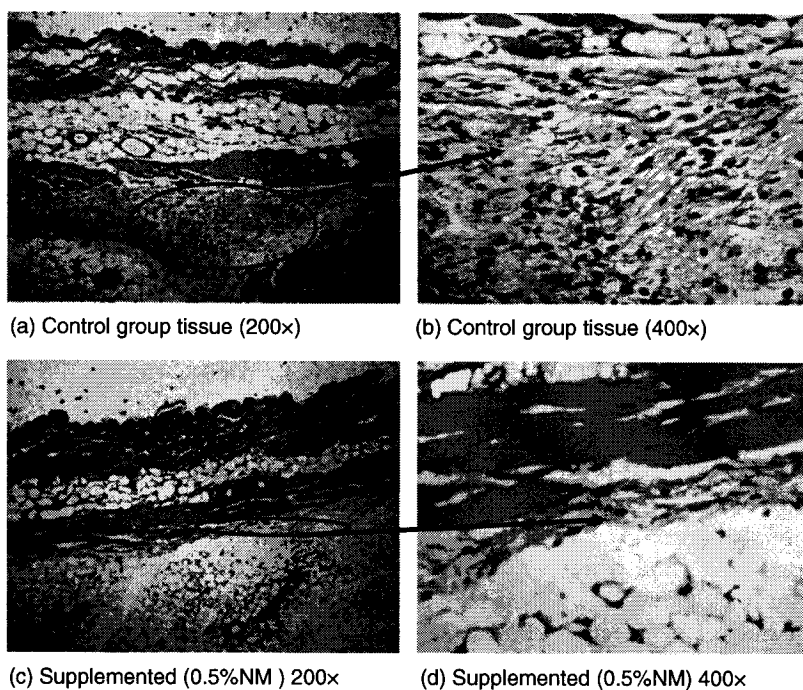


FIGURE 26.2 Effect of NM on bFGF-induced vessel growth in C57BL/6J female mice.

sacrificed, skin was excised, fixed, and stained with H&E and by the Masson-Trichrome method—and representative photographs were taken. The test group of mice received NM in the injection mixture and the control mice received just the vehicle. After seven days, red blood cells were abundant within the lumen of numerous vessels in the control mice (Figure 26.2a and Figure 26.2b). In contrast, NM strongly suppressed the bFGF-stimulated angiogenesis in supplemented mice (Figure 26.2c and Figure 26.2d) [23].

26.4 THE EFFECT OF NM ON HUMAN OSTEOSARCOMA MNNG–HOS CELLS IN VIVO

We also tested the nutrient mixture on tumor growth in vivo by implanting human osteosarcoma MNNG–HOS cells in athymic male nude mice and treating one group of mice with nutrient-supplemented (NM 0.5%) Purina mouse chow and the other group with unsupplemented Purina mouse chow.

Human osteosarcoma cells MNNG–HOS (ATCC, Rockville, MD) were maintained in MEM culture, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY. At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a concentration of

