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

M. Waheed Roomi, V. Ivanov, T. Kalinovsky, A. Niedzwiecki, and M. Rath

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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 angiopoeitn-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, thromobspondin, 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 decreases 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.

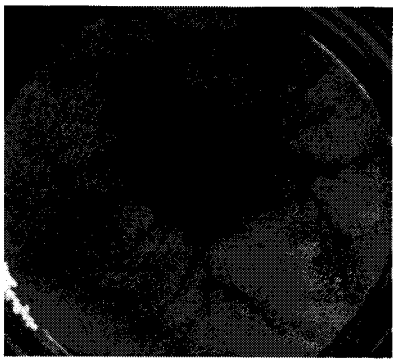
26.2 COMPOSITION OF THE NUTRIENT MIXTURE

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.

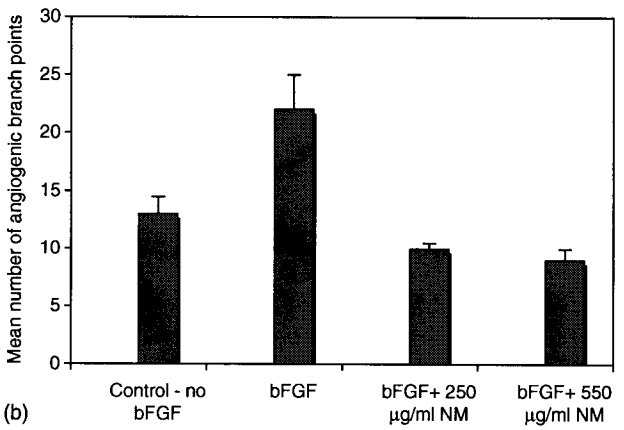
We formulated and tested NM because we were looking at the multiple effects of cancer inhibition at different stages of cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation; the amino acids lysine and proline are necessary for formation of collagen 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 μg/ml EGCG to that of 50 μg/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.



(a)



(b)

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 μl of recombinant bFGF at a concentration of 1.0 μg/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 μg/embryo) in a total volume of 100 μl. At the end of a 3-day incubation period, the embryos were sacrificed and the CAMs were ressected 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

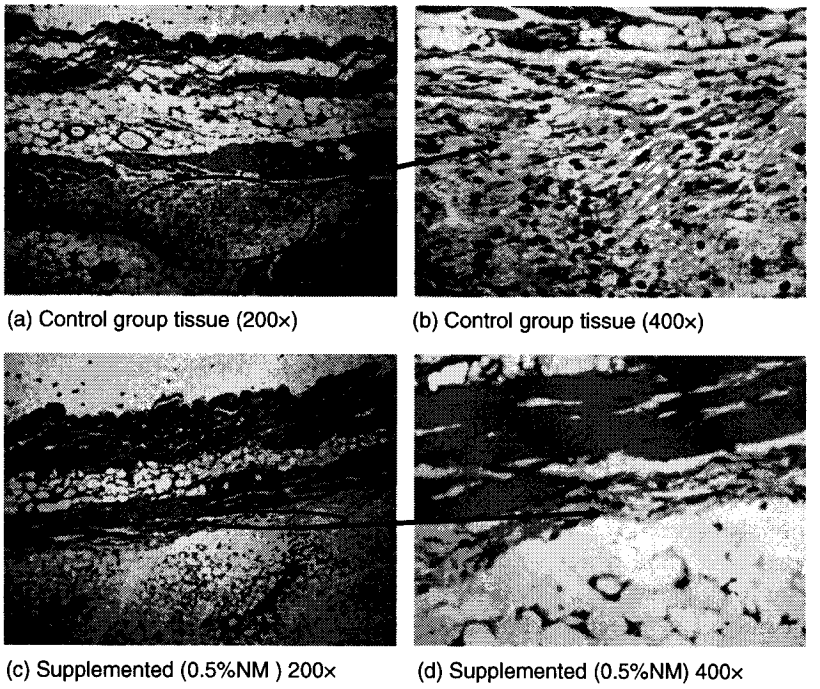


FIGURE 26.2 Effect of NM on bFGF-induced vessel growth in C57BL/6J female mice.

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 μg/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

3×10^6 cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA) for inoculation.

Male athymic nude mice (NCr–nu/nu), approximately six weeks of age on arrival, (Simonsen Laboratories, Gilroy, CA) were maintained in microinsulator cages under pathogen-free conditions on a 12-hour light/12-hour dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for a week, the mice were inoculated with 3×10^6 human osteosarcoma MNNG–HOS cells in 0.2 ml of PBS and 0.1 ml of Matrigel. After implantation, the 12 mice were randomly divided into two groups of six mice. From day one, mice from Group A were fed a Purina mouse chow diet and those in Group B were fed a Purina mouse chow diet supplemented with 0.5% NM. After four weeks, the mice were sacrificed, tumors were excised, measured, weighed, fixed in 10% (v/v) buffered formalin, and processed for histology.

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4–5 μm. Sections were deparaffinized through xylene and a graduated alcohol series to water, and incubated for 5 min in aqueous 3% hydrogen peroxide to block endogenous peroxidase. Histological sections were stained with hematoxylin and eosin (H&E) stain for evaluation using a standard light microscope. Immunochemical studies were performed on formalin-fixed, paraffin-embedded sections. Standard immunohistochemical staining procedures were used for staining antibodies. After deparaffinization and appropriate epitope retrieval, the sections were incubated with primary antibody. Detection was by biotinylated goat anti-mouse antibodies followed by streptavidin conjugated to horseradish peroxidase with the use of diaminobenzidine as the chromogen. Polyclonal rabbit anti-human antibodies used for MMP-9 and VEGF were obtained from Santa Cruz Biotechnology, Inc., CA, and from Sigma.

The results provided further evidence of the significant cancer protecting effect of this specific nutrient mixture in nude mice. Nude mice on nutrient-supplemented diet with implants of the highly metastatic human osteosarcoma MNNG–HOS cells developed significantly smaller tumors (by 53%, $p = 0.0001$) and less vascular ones than did the tumors grown in the control group of nude mice [23] (see Figure 26.3a through Figure 26.3d). Furthermore, immunohistochemical analysis of these tumors demonstrated lower VEGF staining, an indicator of angiogenesis, in nutrient-supplemented animals compared to the control group of mice. This was accompanied by lower cytoplasmic staining for MMP-9 in tumor tissues taken from nutrient-supplemented mice than those fed the standard diet, indicating decreased matrix degradation (Figure 26.4) [23].

26.5 THE EFFECT OF NM ON IN VITRO STUDIES IN HUMAN OSTEOSARCOMA U2OS CELLS

We also tested the effect of the nutrient mixture on tumor cells in culture, with an eye towards the invasive and angiogenic potential. Human osteosarcoma cells U2OS (ATCC) were grown in McCoy medium supplemented with 10% fetal bovine serum,

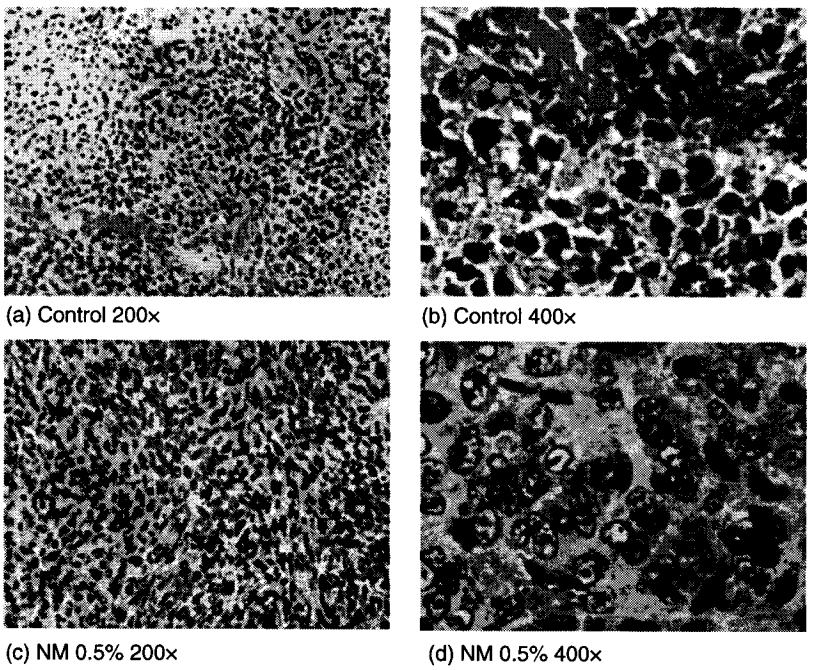


FIGURE 26.3 Effect of NM on tumor tissue vascularity of athymic nude mice receiving osteosarcoma MNNG xenografts.

penicillin (100 U/ml), and streptomycin (100 mg/ml). 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 NM dissolved in media and tested at 0, 10, 100, 500, and 1000 μg/ml in triplicate at each dose. A group of cells were also treated with PMA 200 ng/ml. The plates were then returned to the incubator.

26.6 EFFECT OF NM ON HUMAN OSTEOSARCOMA

26.6.1 U2OS MMP Activity: Gelatinase Zymography

Matrix metalloproteinase 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 $CaCl_2$, 5 μM $ZnCl_2$, and pH 7.5, and then stained with Coomassie Blue R 0.5% for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined. Zymography demonstrated secretion of MMP-2 and MMP-9 by human osteosarcoma U2OS cells

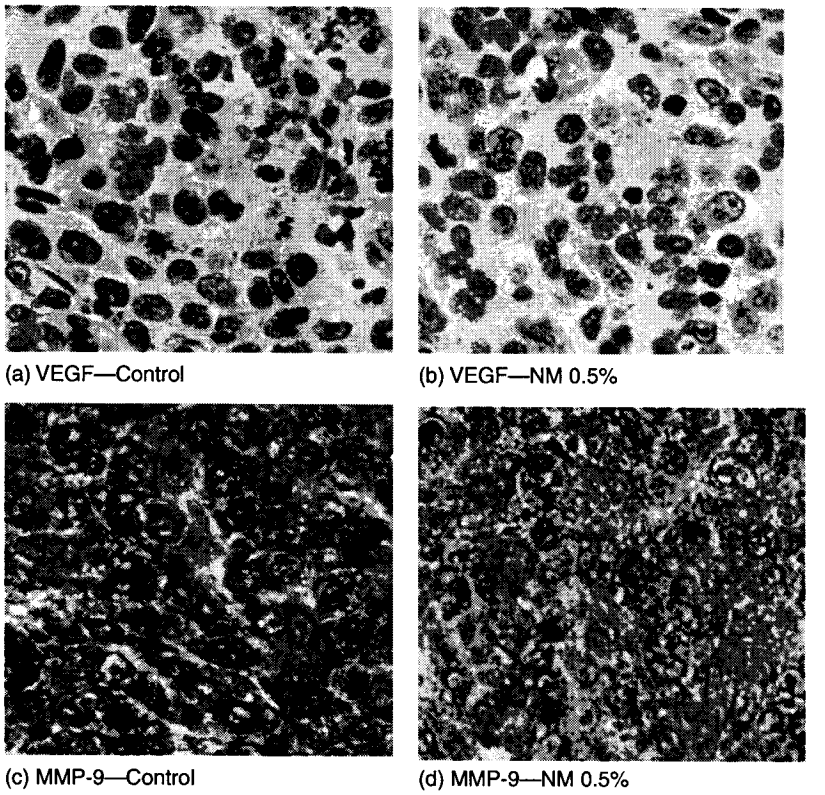


FIGURE 26.4 Effect of NM on tumor tissue of athymic nude mice receiving osteosarcoma MNNG xenografts: immunohistochemistry.

with significantly increased MMP-9 activity in PMA (200 ng/ml) treated osteosarcoma cells. The nutrient mixture inhibited the expression of both MMPs in a dose-dependent fashion with virtual total inhibition at 500 μg/ml concentration (Figure 26.5) [23].

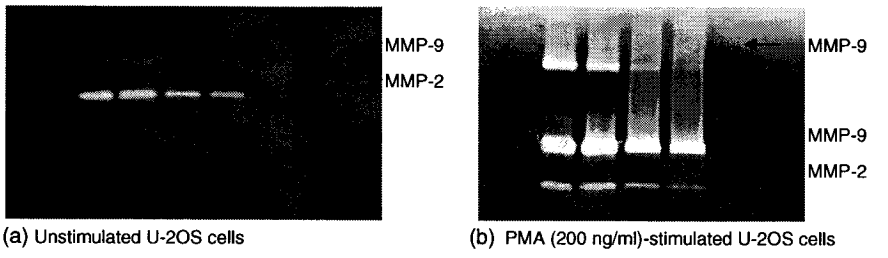


FIGURE 26.5 Effect of NM on MMP secretion by human osteosarcoma U2OS cells.

26.7 EFFECT OF NM ON HUMAN OSTEOSARCOMA U2OS CELL MATRIGEL INVASION

Invasion studies were conducted using Matrigel™ (Becton Dickinson) inserts in 24-well plates. Suspended in medium, osteosarcoma U2OS 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% CO_2 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 and visually counted under the microscope. Invasion of osteosarcoma cells U2OS through Matrigel was reduced by 74% at 50 μg/ml and totally inhibited at 100 μg/ml ($p=0.003$) (see Figure 26.6) [23].

26.8 EFFECT OF NM ON HUMAN OSTEOSARCOMA U2OS SECRETION OF VEGF, IL-6, IL-8, FGF, AND TGFB

Nutrient mixture was also found to inhibit the secretion of proangiogenic factors by osteosarcoma U2OS: VEGF, IL-6, IL-8, bFGF, and TGF-β (Table 26.1) [23]. These factors, which have been identified in various settings of physiologic and pathologic angiogenesis, affect the endothelium directly or indirectly by activation of surrounding cells to produce other factors with proangiogenic activity or modulation of receptors/receptor activities [25]. Conditioned media were collected after confluent cell culture incubation for 24 h in serum-free medium with the indicated supplements. Triplicate samples were pooled and the level of respective protein was measured in duplicate using an immunoassay kit (BioSource International and Quantikine, R&D) according to the manufacturer's protocol. Values are expressed as mean in percentage units of unstimulated control.

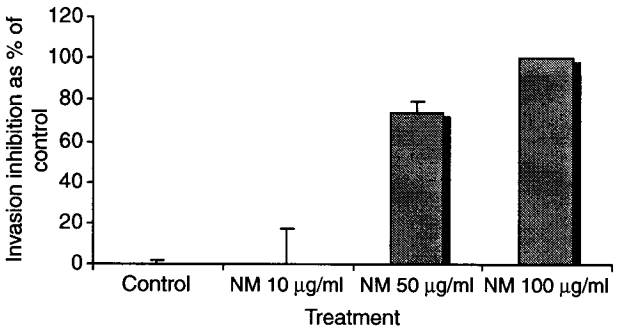


FIGURE 26.6 Effect of NM on human osteosarcoma U2OS invasion and migration through Matrigel.

TABLE 26.1
Effect of the Nutrient Mixture (NM) on Angiogenesis Mediator Secretion by Osteosarcoma U2OS Cells Treated with PMA (200 ng/ml)

Pro-Angiogenic Factor	Factor Level in PMA-Treated U2OS Cells as % of Untreated Control	Factor Level in PMA and + NM (100 μg/ml)-Treated U2OS Cells as % of Untreated Control	NM Reduction of Factor Secretion (%)
VEGF	172	100	72
Interleukin-6	560	100	460
Interleukin-8	4800	300	4500
TGF-β	140	29	111
FGF	56	11	45

26.9 EFFECT OF THE NUTRIENT MIXTURE ON ENDOTHELIAL CELLS

Tumor growth is dependent upon angiogenesis and vascular remodeling. Endothelial cell activation is the first process to occur under pathological and physiological conditions. To initiate formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade the stroma of neighboring tissue. Therefore, we also investigated the effect of the NM on endothelial cell angiogenic parameters.

Human umbilical vein endothelial cells (HUVECs) obtained from ATCC 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 NM dissolved in media and tested at 0, 10, 100, 500, and 1000 μg/ml in triplicate at each dose. The plates were then returned to the incubator.

26.10 EFFECT OF NM ON HUVEC CAPILLARY TUBE FORMATION

The inhibitory effect of NM on capillary tube formation (in vitro differentiation) was assessed by exposure to NM of HUVECs on Matrigel. Unpolymerized Matrigel was placed in wells (300 μl/well) of a 24-well tissue culture plate and allowed to polymerize for an hour at 37°C. Human umbilical vein endothelial 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 concentration. After 24 h of 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. The

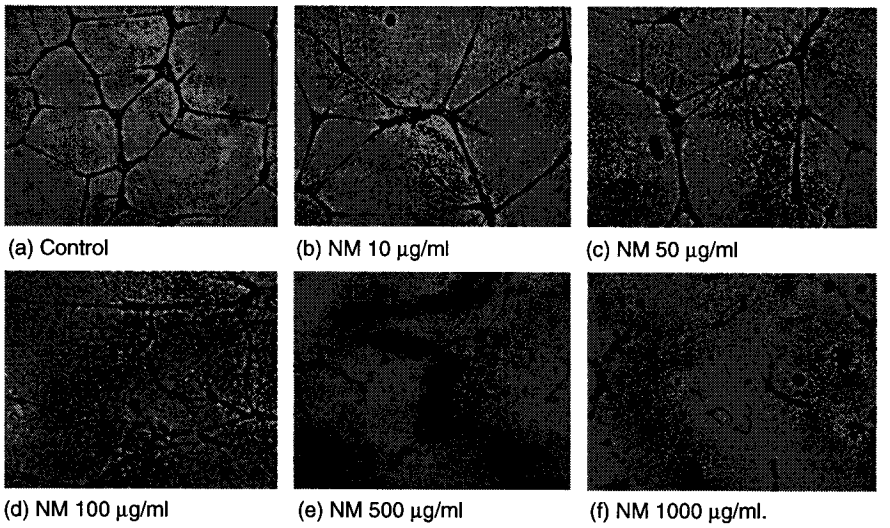


FIGURE 26.7 Effect of the nutrient mixture (NM) on HUVEC capillary tube formation assay (H&E, 100x).

inhibitory effect of NM on capillary tube formation (in vitro differentiation) was assessed by exposure to NM of HUVECs on Matrigel.

After 22 h, the control showed HUVEC-formed hollow tubes on Matrigel in contrast to the NM treated HUVECs, which demonstrated dose-dependent inhibition of capillary tube formation with complete disruption of capillary tubes at 1000 µg/ml NM (Figure 26.7a through Figure 26.7f H&E 100x) [26]. 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 [27]. Capillary tube formation is a complex process requiring cell-matrix interactions and inter-cellular communications, as well as cell motility. The inhibitory effect of NM on HUVEC vascular network formation in light of the other in vitro studies indicates NM inhibits attachment, migration, and invasion of endothelial cells.

26.11 EFFECT OF NM ON HUVEC PROLIFERATION: MTT ASSAY 24 H

Nutrient effects on HUVEC proliferation were evaluated by an 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. 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 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%. The nutrient mixture had no significant effect on HUVEC proliferation [26].

26.12 EFFECT OF NM ON HUVEC MORPHOLOGY (H&E)

Human umbilical vein endothelial cell morphology was unchanged even at the highest concentration (Figure 26.8) [26].

26.13 EFFECT OF NM ON HUVEC MMP EXPRESSION: GELATINASE ZYMOGRAPHY

Matrix metalloproteinase 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 $CaCl_2$, 5 µM $ZnCl_2$, and pH 7.5, and then stained with Coomassie Blue R 0.5% for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined.

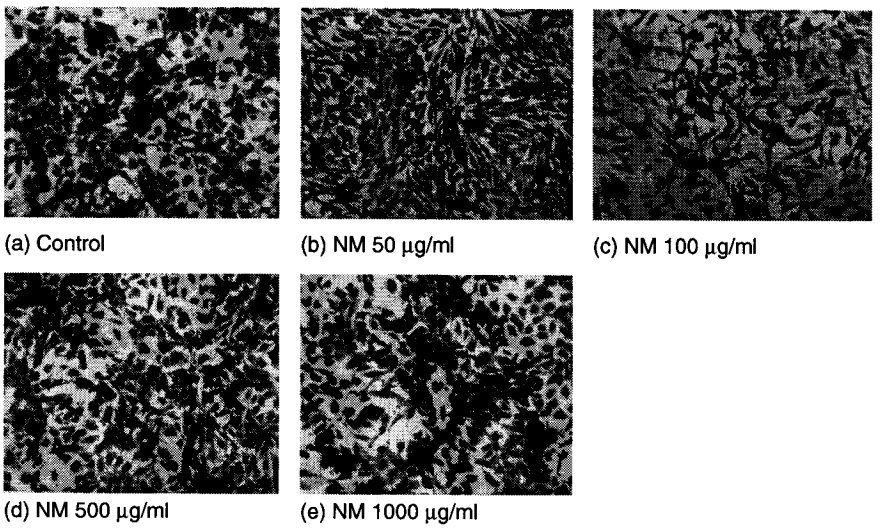


FIGURE 26.8 Effect of the nutrient mixture (NM) on HUVEC morphology.

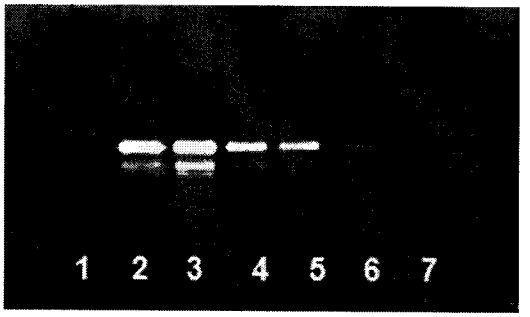
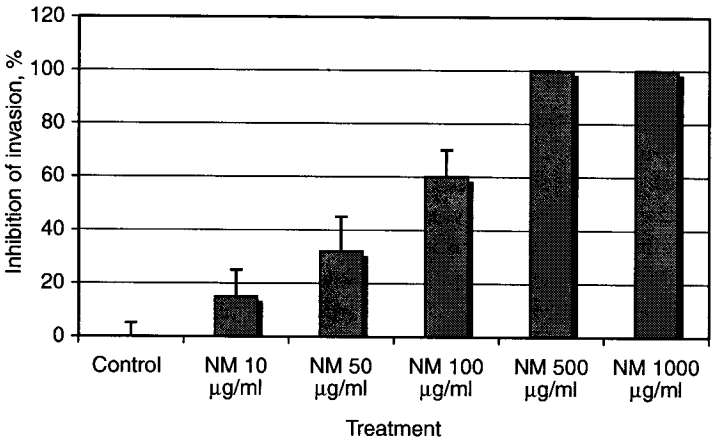


FIGURE 26.9 Effect of NM on HUVEC MMP-2 secretion; Legend: 1-Markers, 2-Control, 3–7 NM 10, 50, 100, 500, 1000μg/ml.



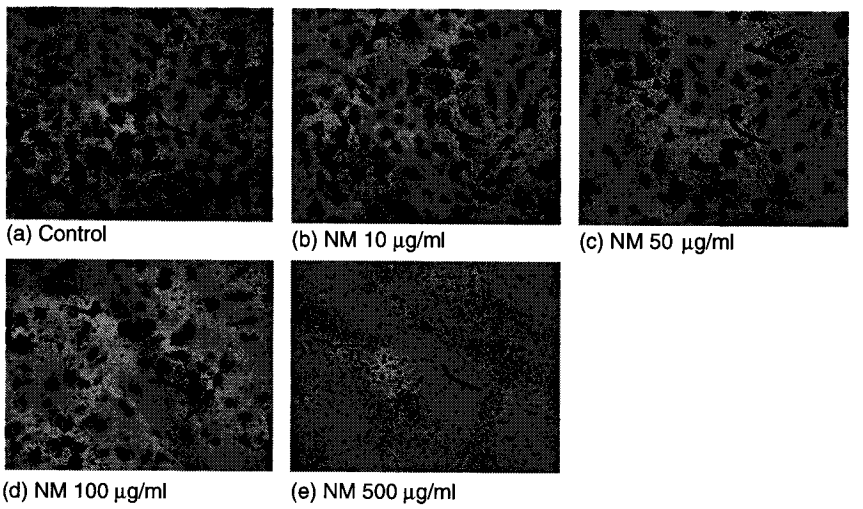


FIGURE 26.10 Effect of NM on HUVEC Matrigel invasion.

Zymography demonstrated expression of MMP-2 by HUVECs. Nutrient mixture inhibited the expression of MMP-2 in a dose-dependent fashion with virtual total inhibition at 500 μg/ml concentration (Figure 26.9) [26].

26.14 EFFECT OF NM ON HUVEC MATRIGEL INVASION

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 for 24 h in a culture incubator equilibrated with 95% air and 5% CO_2. 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

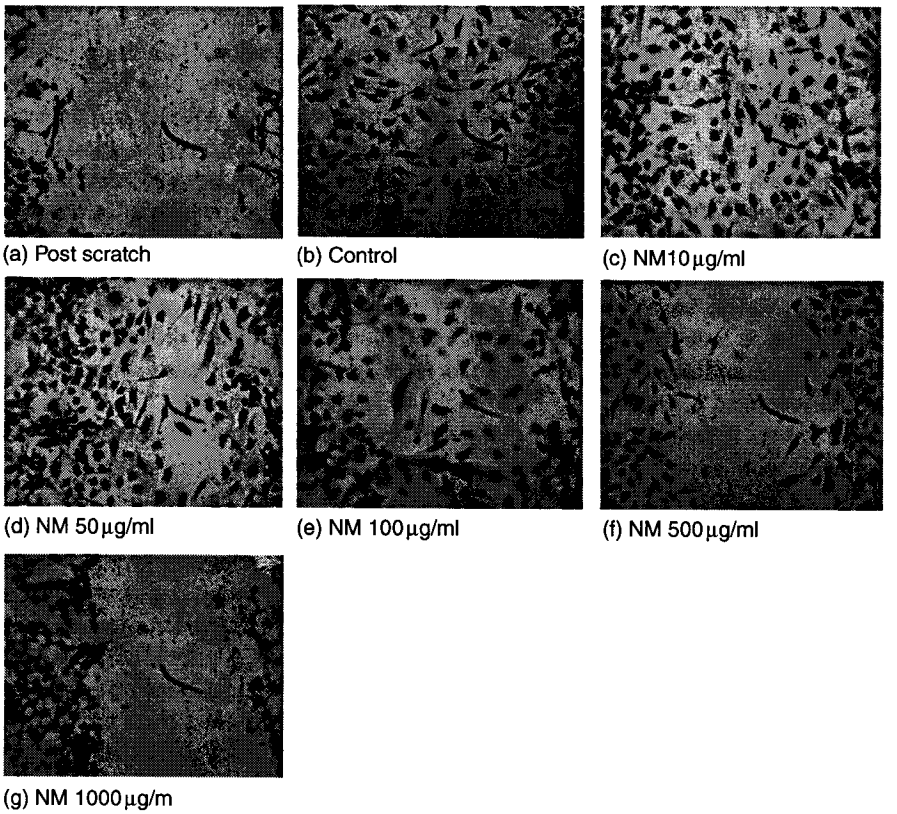


FIGURE 26.11 Effect of NM on HUVEC migration: scratch test.

and Eosin and visually counted under the microscope. Invasion of HUVECs through Matrigel was reduced by 60% at 100 μg/ml and totally inhibited at 500 μg/ml ($p<0.0001$) (Figure 26.10) [26].

26.15 EFFECT OF NM ON HUVEC MIGRATION

Human umbilical vein endothelial cell migration was investigated by making a 2 mm wide single uninterrupted scratch from top to bottom of the culture plates at near confluence. 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, then fixed and stained with H&E. Photomicrographs 100x were taken. Nutrient mixture reduced HUVEC migration by a scratch test in a dose-dependent fashion with total inhibition at 500 μg/ml concentration, as shown in Figure 26.11 [26].

26.16 DISCUSSION

A promising therapeutic approach to cancer is through simultaneous targeting of universal pathomechanisms involved in cancer progression. Inhibiting matrix degradation and optimizing ECM structure and its integrity can curb cancer invasiveness and angiogenesis. Control of ECM proteolytic activity provides an opportunity to address common mechanisms of metastasis, angiogenesis, and tumor growth. Rath and Pauling [6] suggested targeting plasmin-mediated mechanisms with nutritional components, such as lysine and its analogues, which interfere with plasminogen activation into plasmin by tissue plasminogen activator (tPA), by binding to plasminogen active sites and consequently affecting the plasmin-induced MMP activation cascade. Expression of urokinase-type plasminogen activator inhibitor type one by human prostate carcinoma cells was shown to inhibit angiogenesis and metastasis to lung and liver in an athymic mouse model [28]. Lysine-mediated effects on the ECM include increased connective tissue strength and stability. It is well known that the synthesis and structure of collagen fibrils depends upon hydroxylation of proline and lysine residues in collagen fibers, catalyzed by ascorbic acid. Lysine importance in collagen structure and synthesis relates to it being the most abundant amino acid in collagen. Both ascorbic acid and lysine are not produced in the human body; therefore, sub-optimal levels of these nutrients are possible in various pathological stages and through deficient diets.

The inhibitory effects of the individual nutrients composing the nutrient mixture have been reported in both clinical and experimental studies. Ascorbic acid has been reported to have cytotoxic, antimetastatic, and anti-angiogenic actions on malignant cell lines [29–32]; in addition, low levels of ascorbic acid have been reported in cancer patients [33–35]. Green tea extract is a potent anticancer agent that has been reported to have antitumorigenic and anti-angiogenic effects against human cancer cell lines [36–41]. 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 [21].

Because of the growing number of agents tested for anti-angiogenic properties, the U.S. National Cancer Institute (NCI) uses a classification system that categorizes anti-angiogenic drugs into five groups: (1) agents that inhibit endothelial cells directly; (2) agents that block activators of angiogenesis; (3) agents that block ECM breakdown; (4) agents that inhibit endothelial-specific integrin signaling; and (5) agents with non-specific mechanisms of action. Many MMP inhibitors are also anti-angiogenic agents. 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 [42].

Tumor cells are rapidly changing because of their genetic instability, heterogeneity, and high rate of mutation. We postulate that cancer development and progression can be controlled only through a multi-targeted approach, in contrast to the application of agents selected for a highly specific metabolic target. The results of this and our previous studies indicate that such multifaceted approaches targeting the mechanisms that are common to all types of cancer cells can be achieved through nutrient synergy. We demonstrated the inhibitory effects of this nutrient synergy on cancer cell invasion, proliferation, and angiogenesis executed through various mechanisms. We have shown that this nutrient mixture affects MMPs, can induce apoptosis, and modulates the effects of various growth factors.

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 the formulation of green tea extract, lysine, proline, and ascorbic acid is an excellent candidate for adjunctive therapeutic use in the treatment of metastatic cancer, as it inhibits MMP expression and invasion and angiogenesis without cytotoxic effects.

REFERENCES

1. Folkman, J., What is the evidence that tumors are angiogenesis dependent?, *J. Natl. Cancer Inst.*, 82, 4–6, 1990.
2. Fang, W., Li, H., Kong, L., Niu, G., Gao, Q., Zhou, K., Zheng, J., and Wu, B., Role of matrix metalloproteinases (MMPs) in tumor invasion and metastasis: serial studies on MMPs and TIMPs, *Bejing D Xue Bao*, 4, 441–443, 2003.
3. Wahl, M. R., Moser, T. L., and Pizzo, S. V., Angiostatin and antiangiogenic therapy in human disease, *The Endocrine Society*, 59, 73–104, 2004.
4. Canfield, A. E., Schor, A. M., Schor, S. L., and Grant, M. E., The biosynthesis of extracellular matrix components by bovein retinal endothelial cells displaying distinctive morphological phenotypes, *Biochem. J.*, 235, 375–383, 1986.
5. Cao, Y., Veitonmaki, N., Keough, K., Cheng, H., Lee, L. S., and Zurakowski, D., Elevated levels of angiostatin and plasminogen/plasmin in cancer patients, *Intl. J. Mol. Med.*, 5, 547–551, 2000.

6. Rath, M. and Pauling, L., Plasmin-induced proteolysis and the role of apoprotein(a), lysine and synthetic analogs, *Ortho. Med.*, 7, 17–23, 1992.
7. 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.
8. Roomi, M. W., Ivanov, V., Kalinovsky, T., Niedzwiecki, A., and Rath, M., Antitumor effect of nutrient synergy on human osteosarcoma cells U2OS, MNNG–HOS and Ewing's sarcoma SK-ES.1, *Oncol. Rep.*, 13 (2), 253–257, 2005.
9. Roomi, M. W., 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 (2), 129–138, 2005.
10. Roomi, M. W., 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 (2), 40–43, 2004.
11. Roomi, M. W., 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 (2), 97–102, 2005.
12. Roomi, M. W., 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 (3), 421–445, 2005.
13. Roomi, M. W., 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 (1), 179–183, 2005.
14. Roomi, M. W., 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.
15. Maramag, C., Menon, M., Balaji, K. C., Reddy, P. G., and Laxmanan, S., Effect of vitamin C on prostate cancer cells in vitro: effect of cell number, viability and DNA synthesis, *Prostate*, 32, 188–195, 1997.
16. Hare, Y., *Green Tea: Health Benefits and Applications*, Marcel Dekker, New York, Basel, 2001.
17. Kawakami, S., Kageyama, Y., Fujii, Y., Kihara, K., and Oshima, H., Inhibitory effects of *N*-acetyl cysteine on invasion and MMP-9 production of T24 human bladder cancer cells, *Anticancer Res.*, 21, 213–219, 2001.
18. Morini, M., Cai, T., Aluigi, M. G., Noonan, D. M., Masiello, L., De Flora, S., D'Agostini, F., Albini, A., and Fassina, G., The role of *N*-acetyl cysteine in the prevention of tumor invasion and angiogenesis, *Int. J. Biol. Markers*, 14, 268–271, 1999.
19. Yoon, S. O., Kim, M. M., and Chung, A. S., Inhibitory effects of selenite on invasion of HT 1080 tumor cells, *J. Biol. Chem.*, 276, 20085–20092, 2001.
20. Cooke, J. P. and Dzau, V. J., Nitric oxide synthase: role in the genesis of vascular disease, *Annu. Rev. Med.*, 48, 489–509, 1997.
21. Roomi, M. W., 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 (1–2), 6–11, 2004.

22. Brooks, P. C., Montgomery, A. M., and Cheresh, D. A., Use of the 10-day-old chick embryo model for studying angiogenesis, *Meth. Mol. Biol.*, 129, 257–269, 1997.
23. Roomi, M. W., Roomi, N., Ivanov, V., Kalinovsky, T., Niedzwiecki, A., and Rath, M., Inhibitory effect of a mixture containing ascorbic acid, lysine, proline, and green tea extract on critical parameters in angiogenesis, *Oncol. Rep*, 14(4), 807–815, 2005.
24. Passaniti, A., Taylor, R. M., Phili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R., A simple, quantitative method for assessing angiogenesis and antiangiogenesis agents using reconstituted basement membrane, heparin, and fibroblast growth factor, *Lab. Invest.*, 67, 519–528, 1992.
25. Yoshida, S., Ono, M., Shono, T., Izumi, H., Ishibashi, T., Suzuki, H., and Kuwano, M., Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor α-dependent angiogenesis, *Mol. Cell. Biol.*, 17, 4015–4023, 1997.
26. Roomi, M. W., Ivanov, V., Kalinovsky, T., Niedzwiecki, A., and Rath, M., Antiangiogenic Effects of a Nutrient Mixture on Human Umbilical Vein Endothelial Cells, *Oncol. Rep*, 14(6), 1399–1404, 2005.
27. Friedlander, M., Brooks, P. C., Schaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A., Definition of two angiogenic pathways by distinct α integrins, *Science*, 270, 1500–1502, 1995.
28. Soff, G. A., Sanderowiz, J., Gately, S., Verrusio, E., Weiss, I., Brem, S., and Kwaan, H. C., Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung, liver in an athymic mouse model, *J. Clin. Investig.*, 96, 2593–2600, 1995.
29. Koh, W. S., Lee, S. J., Lee, H., Park, C., Park, M. H., Kim, W. S., Yoon, S. S. et al., Differential effects and transport kinetics of ascorbate derivatives in leukemic cell lines, *Anticancer Res.*, 8, 2487–2493, 1998.
30. Roomi, M. W., House, D., Eckert-Maksic, M., Maksic, Z. B., and Tsao, C. S., Growth suppression of malignant leukemia cell line in vitro by ascorbic acid (vitamin C) and its derivatives, *Cancer Lett.*, 122, 93–99, 1998.
31. Naidu, K. A., Karl, R. C., Naidu, K. A., and Coppola, D., Antiproliferative and proapoptotic effect of ascorbyl stearate in human pancreatic cancer cells: association with decreased expression of insulin-like growth factor 1 receptor, *Dig. Dis. Sci.*, 48 (1), 230–237, 2003.
32. Ashino, H., Shimamura, M., Nakajima, H., Dombou, M., Kawanaka, S., Oikav, T., Iwaguchi, T., and Kawashima, S., Novel function of ascorbic acid as an angiostatic factor, *Angiogenesis*, 6 (4), 259–269, 2003.
33. Anthony, H. M. and Schorah, C. J., Severe hypovitaminosis C in lung-cancer patients: The utilization of vitamin C in surgical repair and lymphocyte related host resistance, *Br. J. Cancer*, 46, 354–367, 1982.
34. Nunez, C., Ortiz, D. E., Apodaca, Y., and Ruiz, A., Ascorbic acid in the plasma and blood cells of women with breast cancer. The effect of consumption of food with an elevated content of this vitamin, *Nutr. Hosp.*, 10, 368–372, 1995.
35. Kurbacher, C. M., Wagner, U., Kolster, B., Andreotti, P. E., Krebs, D., and Bruckner, H. W., Ascorbic acid (vitamin C) improves the antineoplastic activity doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells in vitro, *Cancer Lett.*, 103 (2), 183–189, 1996.
36. Jung, Y. D., Kim, M. S., Shin, B. A., Chay, K. O., Ahn, B. W., Liu, W., Bucana, C. D., Gallick, G. E., and Ellis, L. M., EGCG, a major component of green tea, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells, *Br. J. Cancer*, 84 (6), 844–850, 2001.

37. Koima-Yuasa, A., Hua, J. J., Kennedy, D. O., and Matsui-Yuasa, I., Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors, *Life Sci.*, 73 (10), 1299–1313, 2003.
38. Valcic, S., Timmermann, B. N., Alberts, D. S., Wachter, G. A., Krutzsch, M., Wymer, J., and Guillen, M., Inhibitory effects of six green tea catechins and caffeine on the growth of four selected human tumor cell lines, *Anticancer Drugs*, 7, 461–468, 1996.
39. Mukhtar, H. and Ahmed, N., Tea polypheonols: prevention of cancer and optimizing health, *Am. J. Clin. Nutr.*, 71, 1698S–1720S, 2000.
40. Yang, G. Y., Liao, J., Kim, K., Yurkow, E. J., and Yang, C. S., Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols, *Carcinogenesis*, 19, 611–616, 1998.
41. Metz, N., Lobstein, A., Schneider, Y., Gosse, F., Schleiffer, R., Anton, R., and Raul, F., Suppression of azoxymethane-induced preneoplastic lesions and inhibition of cyclooxygenase-2 activity in the colonic mucosa of rats drinking a crude green tea extract, *Nutr. Cancer*, 38 (1), 60–64, 2000.
42. Griffioen, A. W. and Molema, G., Angiogenesis: potential for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation, *Pharm. Rev.*, 52 (2), 237–268, 2000.