

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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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.

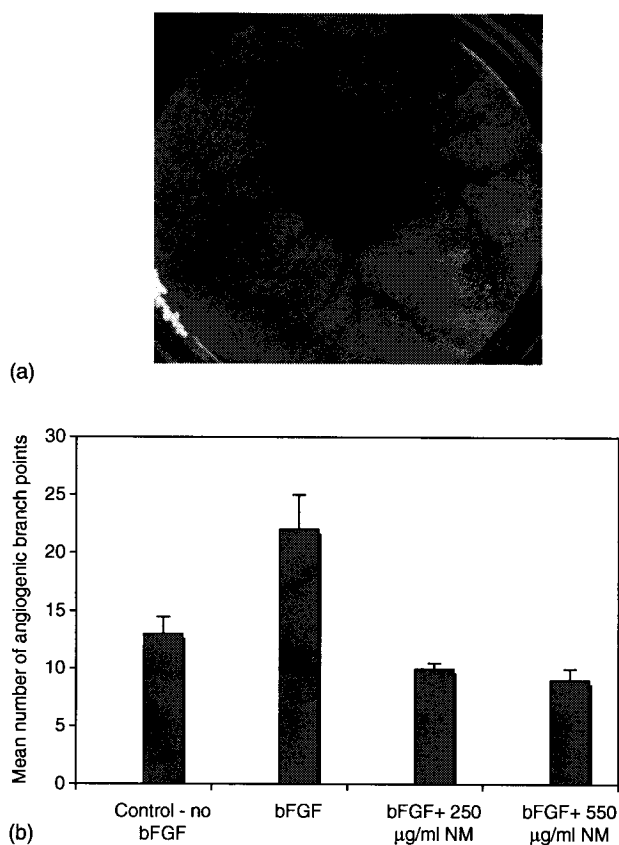


FIGURE 26.1 Effect of NM on bFGF-induced angiogenesis in chick CAM assay.

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

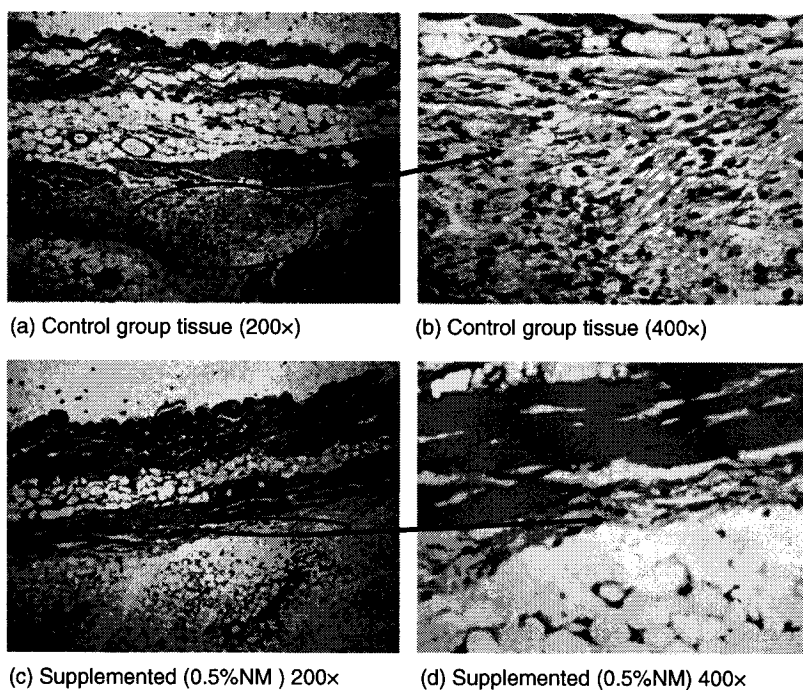


FIGURE 26.2 Effect of NM on bFGF-induced vessel growth in C57BL/6J female mice.

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 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,

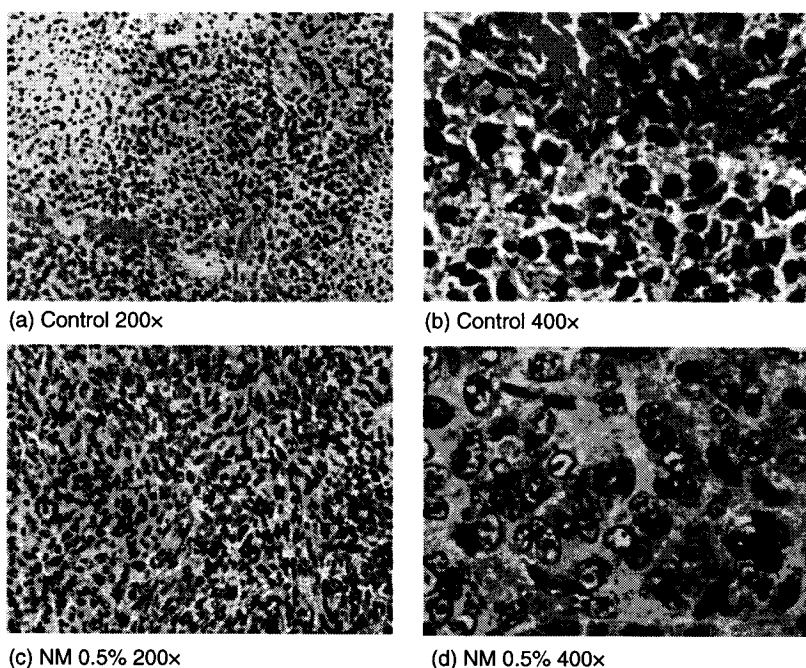


FIGURE 26.3 Effect of NM on tumor tissue vascularity of athymic nude mice receiving osteosarcoma MNNG xenografts.

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% CO₂. 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 CaCl₂, 5 µM ZnCl₂, 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

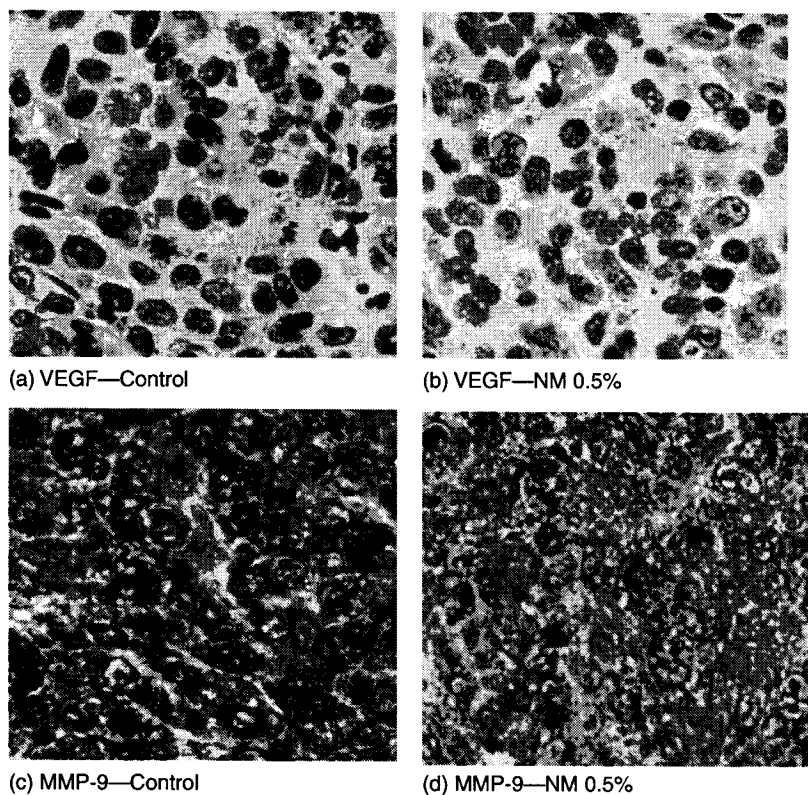
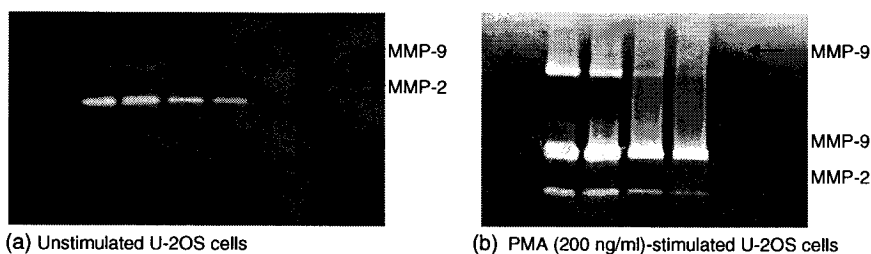


FIGURE 26.4 Effect of NM on tumor tissue of athymic nude mice receiving osteosarcoma MNNG xenografts: immunohistochemistry.

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].



1-Markers, 2-Control, 3-7 Nutrient Mixture 10, 50, 100, 500, 1000 µg/ml

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% CO₂ for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with Hematoxylin and Eosin 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 TGFβ

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.

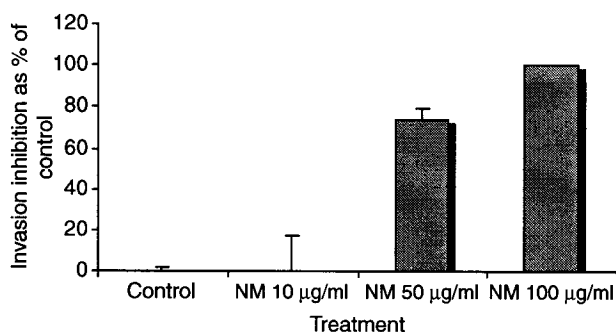


FIGURE 26.6 Effect of NM on human osteosarcoma U2OS invasion and migration through Matrigel.

