Suppression of metastasis of intratesticular inoculation of B16FO melanoma cells by a novel nutrient mixture in male athymic nude mice

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Abstract. Metastasis, commonly to the lung, is the major cause of mortality from testicular cancer. The aim of the present study was to examine the effect of a novel nutrient mixture (NM) containing ascorbic acid, amino acids and green tea extract on the inhibition of melanoma growth and metastasis using a model of intratesticular inoculation of B16FO cells into nude mice. Male athymic mice (n=12), 10-12 weeks of age, were inoculated with $5x10^5$ B16FO melanoma cells in 100 μl of PBS into the right testis, while the left testis was left untreated. Following inoculation, the mice were randomly divided into two groups. The control group (n=6) was fed a regular mouse chow diet and the NM 1% group (n=6) the same diet, but supplemented with 1% NM. Four weeks later the mice were sacrificed and the abdominal cavity was opened. Mice in the control group exhibited extensive metastasis in the peritoneal cavity and severely enlarged right testes and necrotic seminiferous tubules. By contrast, in the NM 1% fed group there was no evidence of peritoneal metastasis in 50% of the animals and mild metastasis in the remaining 50%. The right testes were enlarged and seminiferous tubules in the area of invasion showed evidence of degeneration. No metastasis to the liver, kidney or spleen were evident in either group. However, severe lung metastasis was observed in 2 of 6 mice in the control group and mild metastasis in 2 of 6 mice in the NM 1% group. In conclusion, these results confirm earlier studies and verify the anti-metastatic potential of NM.

Introduction

Testicular cancer (TC) is a relatively rare type of cancer that may often leads to metastasis. Approximately 8,590 new cases are likely to be diagnosed in the United States in 2012, of which 360 individuals are expected to succumb to the disease (1). Although the disease is not common, as the likelihood of developing testicular cancer is approximately 1 in 270, the rate of testicular cancer has been on the increase in the United States and many other countries, with the increase mostly in seminomas (1). Caucasians have 5 times the risk of developing the disease compared to African-Americans and 3 times that of Asian-Americans (1). As yet, no rationale for the increase has been found. TC affects males of all ages although 90% of cases occur in men between the ages of 20 and 54 years. Factors that increase the risk of developing testicular cancer include undescended testicle, Kleinfelder syndrome, family history of testicular cancer, HIV infection, particularly in those with AIDS, carcinoma *in situ*, and cancer of the other testicle (1). TC is considered to be one of the most curable forms of cancer. However, if the cancer has metastasized beyond the lymph nodes, the 5-year survival is reduced to 71% (1). The primary modality of spread of TC is through the lymph node system to the retroperitoneum and in certain cases to other lymph nodes along the mid-line of the body. Spread continues through the bloodstream, common in patients with advanced germ cell tumors or those with choriocarcinoma or embryonal carcinoma elements. The main sites for blood-borne metastatic tumors are the lungs, followed by the liver, bone and brain (2,3).

Metastatic malignant melanoma cells, specifically B16, have been successfully utilized for experimental metastasis to study the effectiveness of anticancer agents, since melanoma cells are extremely aggressive and metastasize to secondary areas of the body, such as lymph nodes, lungs, liver, brain or bone (4). Hart and Fidler (4) studied the role of organ selectivity in the determination of metastatic patterns of B16 melanoma and concluded that the outcome of metastasis was dependent on tumor cell properties and host factors, supporting the 'seed and soil' hypothesis to explain the non-random pattern of cancer metastasis. For example, although the circulatory mode of spread leads to the dissemination of a number of malignant cells, it cannot fully explain the patterns of distribution of numerous tumors, such as the infrequent metastatic development in organs including the spleen or skeletal muscle with highly developed vasculature. In their study, Zeidman

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and Busso (5) reported that tumor cells from different tumors interacted differently with the capillary bed of a given organ, while Sugarbaker *et al* (6) found that tumor cell suspensions from different types of tumors injected into the same site in rats established different patterns of metastases. Experimental data have indicated that melanoma B16 cells preferentially metastasize to specific organs, such as the lungs and liver (4,7,8).

A nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract has demonstrated anticancer activity in a number of human cancer cell lines, inhibiting cancer cell growth, MMP secretion, invasion, metastasis and angiogenesis (9). In a previous study, we demonstrated that NM was effective in inhibiting the pulmonary metastasis of B16FO melanoma cells injected into the tail vein of C57BL/6 mice, especially when nutrients were delivered intravenously or intraperitoneally (7). We also demonstrated the effectiveness of dietary supplementation with NM to prevent experimental hepatic metastasis by studying its effect on the intrasplenic injection of B16FO cells into athymic nude mice (10).

The aim of this study was to investigate the effect of NM on the experimental metastasis of melanoma cells by the intratesticular injection of B16FO cells into male nude mice. This experimental model of metastasis was selected to study the effectiveness of nutrients against metastasis to the lungs and other organs from the testes since melanoma cells are aggressive enough to result in significant metastasis in mice, particularly pulmonary metastasis, a common end organ of metastasis for testicular cancer. Furthermore, the intratesticular model was found to be an effective model for studying mechanisms of metastasis and evaluating treatment strategies due to the stable formation of tumors with metastatic potential (11).

Materials and methods

Cancer cell line and culture. Murine melanoma B16FO cells obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The media and sera used were obtained from ATCC, while the antibiotics (penicillin and streptomycin) were purchased from Gibco-BRL (Long Island, NY, USA).

Animals. Male nude mice, approximately 9-11 weeks of age on arrival, were purchased from Simonsen Laboratories (Gilroy, CA, USA), and kept in microisolator cages under pathogen-free conditions on a 12-h light/dark schedule for a week. The animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

Diet. The regular rodent diet was obtained from Purina Mills (Gray Summit, MO, USA). The NM 1% supplemented diet mix was milled and pressed by Purina Mills, LLC, and generated by Vitatech (Tustin, CA, USA). The NM 1% diet comprised the following in the ratio indicated: 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 (80% polyphenol) 1000 mg; selenium 30 μg; copper 2 mg; manganese 1 mg.

Experimental design. Male athymic mice (n=12), 10-12 weeks of age, were anesthetized by inhalation utilizing isofluorane USP (Abbott Laboratories, Chicago, IL, USA). The right side of the abdomen overlying the testis was sterilely prepped and a skin incision of 1 cm was made to expose the right testis. Mice were inoculated with $5x10^5$ B16FO melanoma cells in 100 μl of PBS into the right testis, while the left testis was left untreated. The cavities were sutured and clamped. After inoculation, the mice were randomly divided into 2 groups. The control group (n=6) was fed regular Purina mouse chow diet, while the mice in the NM 1% group (n=6) were fed the same diet, but supplemented with 1% NM. The quantity of diet provided to the mice was unrestricted, however, the mice consumed, on average, 4 g of their respective diets/day. Thus, the supplemented mice received ~40 mg of NM/day, indicating that they received the following amounts of NM components/day: ascorbic acid 7 mg, L-lysine 10 mg, green tea extract 10 mg, L-proline 7.5 mg, L-arginine 5 mg, N-acetyl cysteine 2 mg, selenium 0.3 μg, copper 0.02 mg, manganese 10 μg. Four weeks later the mice were sacrificed, the abdominal cavity was opened and testes, lungs, kidneys, livers and spleens were excised from all the animals and examined. Since growth of testes in the control animals expanded profoundly into the peritoneum making it difficult to determine the limits of the organs, measurements of volume and weight were not carried out. Growth of melanoma colonies in testicles were evaluated by sectioned tissue. Lung metastasis was evaluated by observation of the melanoma colonies. A control mouse was sacrificed and examined at 1 week. All procedures were performed according to humane and customary care and use of experimental animals and conducted under protocols approved by the Internal Animal Care and Use Committee (IACUC).

Histopathology. Testicular tissues were fixed in 10% buffered formalin, embedded in paraffin and cut at 4-5 microns. Sections were deparaffininzed through xylene and graduated alcohol series to water, and stained with hematoxylin and eosin (H&E) for microscopic evaluation by IDEXX Reference Laboratories.

Results

Melanoma growth in testes and peritoneal metastasis. The mice (6/6) in the control group exhibited extensive metastasis in the peritoneal cavity, which was totally masked by B16FO melanoma cells, in contrast to the NM 1% group, which showed no peritoneal metastasis in 3 mice and mild metastasis in 3 mice. Representative images of the peritoneum in the two groups are shown in Fig. 1. The right testis in the control group was severely enlarged and replaced by invading malignant melanoma cells and the remaining testicular tissue was represented by necrotic seminiferous tubules. The capsular region of the testis was severely infiltrated with a population of mixed cells. By contrast, in the NM 1% group, the testes were slightly enlarged and the seminiferous tubules in the area of invasion showed evidence of degeneration. The left testes of the two groups shows no evidence of melanoma colonies; however, the right testes of the control group of nude mice shows extensive melanoma invasion, while the NM 1% group shows mild invasion of melanoma cells (Fig. 2). Profound enlargement of the right testis was observed in the control group mice. By

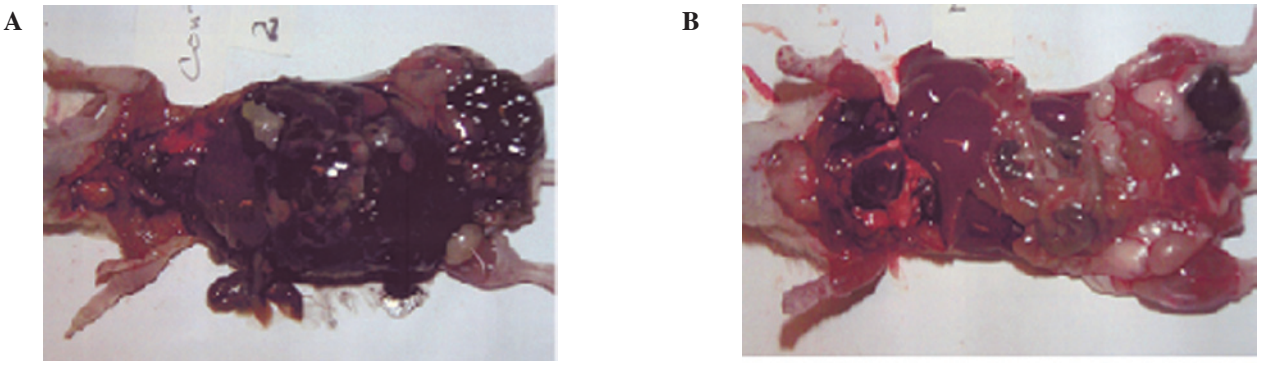

Figure 1. Representative gross images of peritoneal cavities of nude mice injected with B16FO melanoma cells fed the control or NM 1% diet (4 weeks post-injection) are shown. (A) Control diet group and (B) NM 1% diet group.

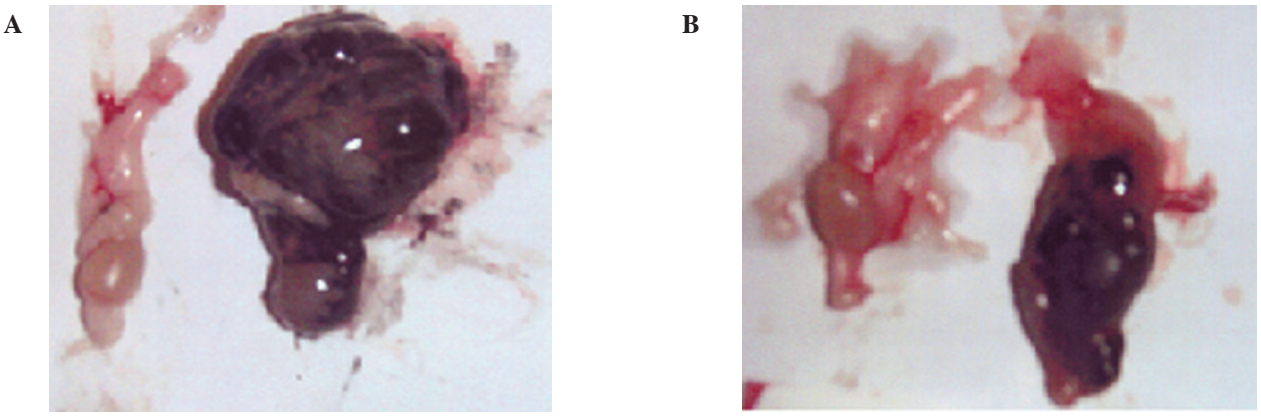

Figure 2. Representative gross images of nude mice testes in the control and NM 1% diet groups are shown. The left testis was untreated and the right testis was injected with B16FO melanoma cells (4 weeks post-injection). (A) Control diet group and (B) NM 1% diet group.

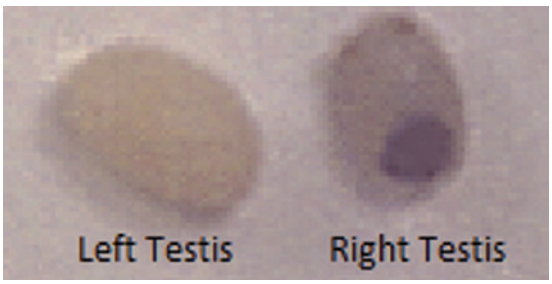

Figure 3. Representative gross photos and photomicrographs of testes from nude mice fed the dontrol diet (1 week post injection). Left testis (untreated), right testis (injected with B16FO cells).

contrast, some enlargement of the right testis was evident in the NM 1% group, although it was much smaller than that observed in the control group. Representative gross images of the left and right testes in the control group at 1 week post-injection are shown in Fig. 3.

Histopathology of representative testicular sections. The right testes in the two groups showed overgrowth of melanoma cells. All of the control mice showed testicular metastasis, while in the NM diet group, only 3 mice showed mild metastasis and 3 mice showed no metastasis. A week after the melanoma injection, the left testis (untreated) of the control mouse was normal, while the right testis showed a focal area of melanoma invasion (Fig. 4). The magnified section (x200) of the right testis (Fig. 4D) shows melanoma cells surrounding the seminiferous tubules at 1 week post-injection. At 4 weeks post-injection, the control mice showed significant testicular invasion by melanoma cells (Fig. 5) in contrast to the less pronounced metastasis in the NM 1% group of mice (Fig. 6).

Lung metastasis. No metastasis to the liver, kidney or spleen was detected in either group. Lung metastasis was observed in 2 of 6 mice in each group. However, severe lung metastasis was observed in the control group, while mild metastasis was detected in the NM 1% group (Fig. 7).

Mean initial and final weights of mice. No significant difference was found between the initial and final mean weights within the two groups. The mean initial weight of the control group was 37.2±1.3 g and the mean final weight was 38.4±1.6 g. The mean initial weight of the NM 1% group was 36.6±1.5 g and the final weight was 36.7±1.1 g.

Discussion

The aim of the present study was to investigate the effect of a nutrient mixture on melanoma B16FO growth and metastasis from intratesticular injection into nude mice, representing the lymphatic and hematogenous dissemination of melanoma malignancy. In our study, supplementation with the nutrient

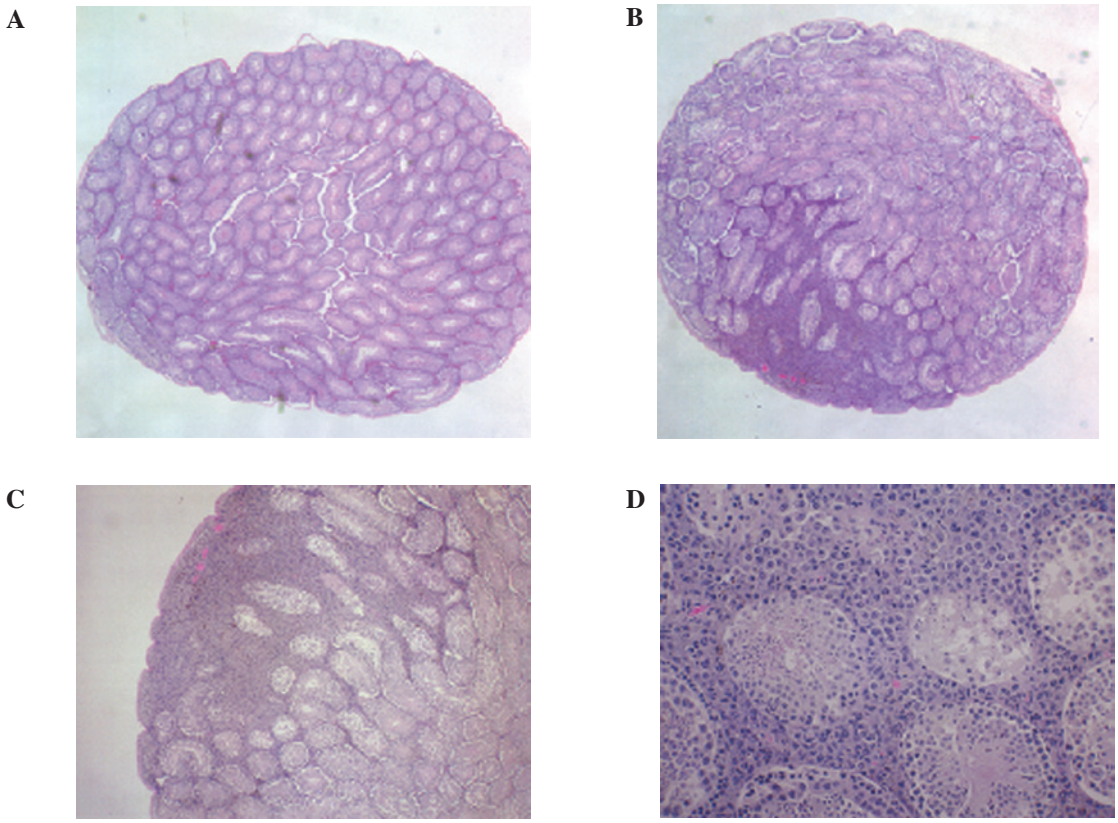

Figure 4. Representative photomicrographs of untreated testis and B16FO-injected testis in the control group (1 week post-injection). (A) Left (untreated) testis, (B) right (B16FO-treated) testis, (C) right (B16FO-treated) testis, x40, showing focal area of melanoma invasion, (D) right (B16FO-treated) testis, x200, showing seminiferous tubules surrounded by melanoma cells.

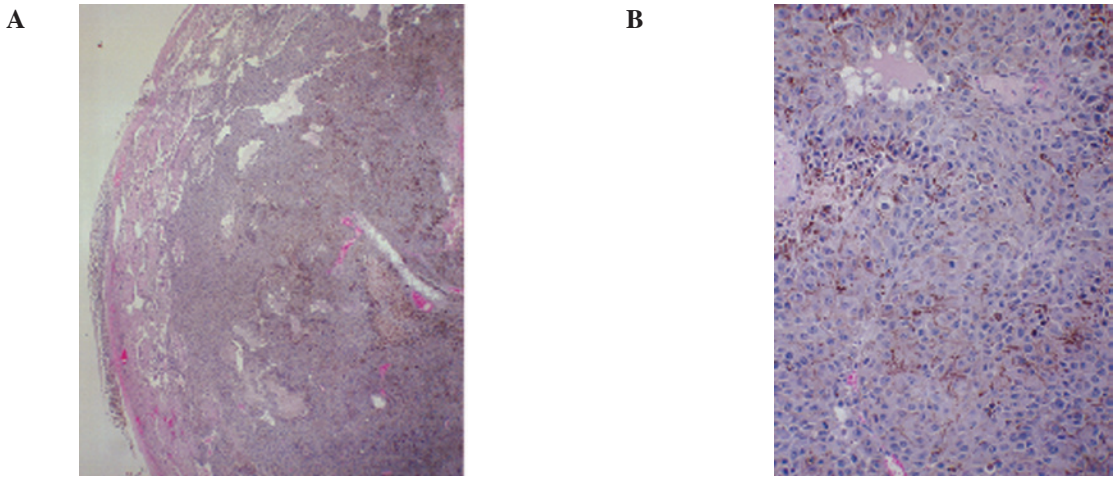

Figure 5. Photomicrographs of B16F0-injected testis in nude mice fed the control diet (4 weeks post-injection) showing invasion of testis by melanoma cells. Magnification: (A) x40, (B) x200.

mixture suppressed B16FO melanoma cell growth in the testes and metastasis to the peritoneum and lungs after intratesticular injection. All of the mice receiving the control diet exhibited extensive metastasis in the peritoneal cavity, in contrast to the NM 1% diet group, which showed no metastasis in 50% of mice and mild peritoneal metastasis in the remaining mice. No metastasis to liver, kidney or spleen was evident in either of the two groups. Lung metastasis was observed in 2 of 6 mice in each group, with severe lung metastasis being observed in the control group and mild metastasis in the NM 1% group.

Notably, the melanoma cells invaded the peritoneum and metastasized to the lungs from the right testes (injection site of B16FO cells), but did not metastasize to the left testes (untreated), suggesting the testes are not common sites for melanoma B16FO cell metastasis. Previously, we showed that intraperitoneal injection of B16FO melanoma cells into C57BL/6 mice demonstrated intraperitoneal growth and ascites, but did not result in metastasis to other organs (10). In regards to testicular tumor growth, we demonstrated that supplementation with dietary NM significantly suppressed murine melanoma

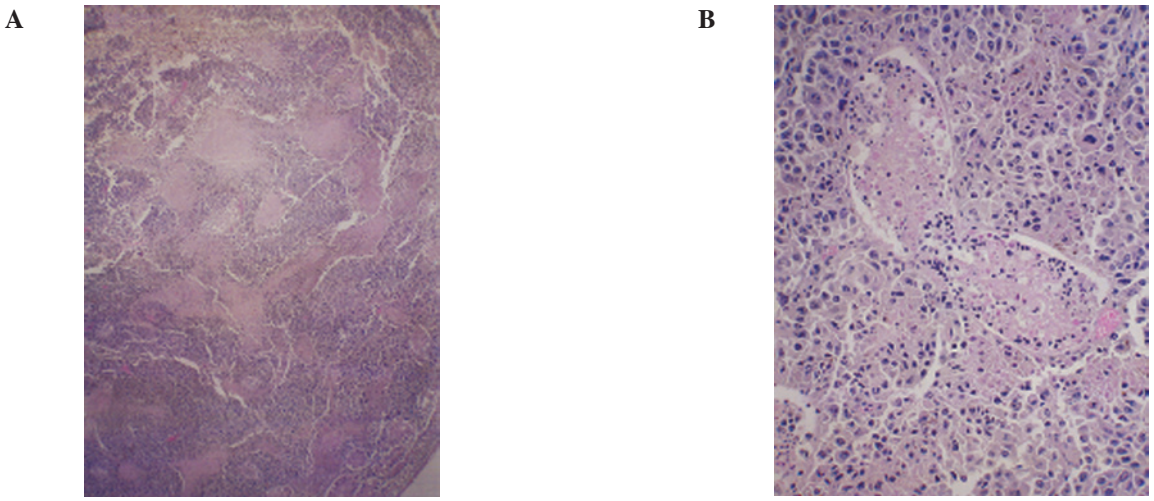

Figure 6. Photomicrographs of B16F0 injected testis in NM 1% diet group of nude mice (4 weeks post-injection) showing melanoma invading testis and degenerating seminiferous tubules. Magnification: (A) x40 and (B) x200.

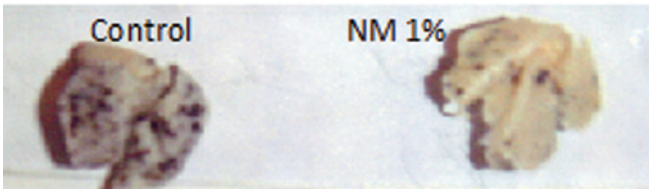

Figure 7. Representative gross images of lungs from nude mice injected with B16FO melanoma cells intratesticularly fed the control (left) or NM 1% diet (right) 4 weeks post injection.

B16FO tumor growth in immune-impaired (athymic) mice. Previous *in vitro* studies have demonstrated significant inhibition of melanoma B16FO and A2058 cell proliferation and strong induction of apoptosis at 500 μg/ml NM, suggesting that inhibition of tumor growth was due probably to induction of apoptosis (12). These findings are in agreement with our *in vivo* findings that exposure of melanoma cells for 18 h to NM before injecting them into mice completely prevented the formation of metastatic lung tumor modules (7).

Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a critical role in the formation of tumors and metastasis (13). Findings of studies have shown that highly metastatic melanoma and other cancer cells secrete higher amounts of MMPs as compared to poorly metastatic cells, demonstrating that the invasive and metastatic abilities of these cancer cells correlate with MMP expression, particularly MMP-9 and -2 (14-18). Type IV collagenases MMP-2 and -9 have been the focus of research as type IV collagen is a major structural protein for ECM and basement membrane, and MMP-2 and -9 expression is associated with cancer cell invasion and elevated in a variety of malignancies (19,20). Previous *in vitro* studies have shown that NM significantly inhibited melanoma and other cancer cell MMP-2 and -9 secretion and Matrigel invasion (21). In addition, ECM synthesized by normal fibroblasts treated with NM exhibited increased stability and significantly reduced the osteosarcoma cell growth rate, invasive activity (MMP-2 and -9 secretion and Matrigel invasion) and adhesion to collagen I and other substrates, suppressing tumor growth independently of the immune system function and inhibiting critical steps in cancer metastasis (22).

Rath and Pauling (23) suggested the use of nutritional components, such as vitamin C and lysine and lysine analogues to target plasmin-mediated connective tissue degradation as a universal approach to controlling common pathomechanisms in cancer progression. Lysine interferes with the activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, thereby affecting the plasmin-induced MMP activation cascade (23). Subsequent studies have confirmed this approach and resulted in identifying a novel formulation (NM) comprising lysine, ascorbic acid, proline and green tea extract, and other micronutrients that have shown significant anticancer activity against a large number (~40) of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both *in vitro* and *in vivo* (9).

NM is a mixture of nutrients that addresses critical physiological targets in cancer progression and metastasis, such as ECM integrity and MMP activity. Optimal ECM formation and structure is dependent upon adequate supplies of ascorbic acid and the amino acids lysine and proline, which ensure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine, a natural inhibitor of plasmin-induced proteolysis, is crucial in supporting ECM stability (23,24). Green tea extract has been shown to be a potent agent in controlling cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (25-29). N-acetyl cysteine has been observed to inhibit MMP-9 activity (30) and invasive activities of tumor cells (31). Selenium inhibits MMP secretion and tumor invasion (32), as well as migration of endothelial cells through ECM (31). In addition to addressing ECM properties, some nutrients are critical in inducing cancer cell death. Findings of a previous study confirmed that ascorbic acid inhibits cell division and growth through the production of hydrogen peroxide (33). Since arginine is a precursor of nitric oxide (NO), any deficiency of arginine is capable of limiting the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells (34).

In conclusion, the results of the present study have shown that the nutrient mixture was effective in significantly reducing melanoma B16FO cell testicular tumor growth and peritoneal and lung metastasis in male nude mice injected with melanoma cells intratesticularly. These findings together with our earlier results clearly indicate the anticancer potential of NM. Furthermore, use of the nutrient mixture is not likely to pose any toxic effect clinically, especially in the relevant doses, as demonstrated by *in vivo* safety studies. During an *in vivo* study on possible toxicity from NM, we found that NM did not have any adverse effect on vital organs, such as the heart, liver and kidney, nor on the associated functional serum enzymes (35).

Acknowledgements

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INHIBITION OF PULMONARY METASTASIS OF MELANOMA B16FO CELLS IN C57BL/6 MICE BY A NUTRIENT MIXTURE CONSISTING OF ASCORBIC ACID, LYSINE, PROLINE, ARGININE, AND GREEN TEA EXTRACT

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☐ *The authors investigated the effect of a nutrient mixture (NM) on lung metastasis by B16F0 melanoma cells in C57BL/6 female mice. Mice were divided into equal groups (1 to 6) and injected via tail vein with B16F0 cells (groups 1 to 4), B16FO cells pretreated with NM (group 5), or saline (group 6). Groups 1, 3, 4, 5, and 6 were fed the control diet and group 2 the 0.5% NM supplemented diet. Groups 3 and 4 received NM intraperitoneally (IP) and intravenously (IV), respectively. Two weeks later, pulmonary metastatic colonies were counted. Pulmonary colonization was reduced by 63% in mice supplemented with NM diet, by 86% in mice receiving NM by IP and IV injections, and completely inhibited in mice injected with melanoma cells pretreated with NM. These results show that NM is effective in inhibiting the metastasis of B16FO melanoma cells.*

Keywords antitumor effect, in vivo, malignant melanoma, metastasis, nutrient mixture

Tumor metastasis is a major reason for treatment failure in cancer patients. Though often curable in its early stages, melanoma may metastasize to other areas of the body, such as lymph nodes, lungs, liver, brain, or bones. Metastatic malignant melanoma is an extremely aggressive cancer with no current viable therapy, causing the most skin cancer-related deaths [1]. Thus any treatment that prevents or slows the spread of malignant cells may reduce progress of the disease and improve patient prognosis.

Invasion of host tissues is a hallmark feature of metastasis, which requires alterations in tumor cell adhesion, cell migration, and proteolytic degradation of the extracellular matrix (ECM) [2]. Tumor cells enter blood vessels or the lymphatic system by invasion of the ECM, then lodge in distant

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capillaries and grow secondary metastases in the new environment [3]. Furthermore, extensive vascularization (angiogenesis) must occur if a tumor mass is to grow over 2 mm in diameter. Angiogenesis not only allows the tumor to increase in size, but it also facilitates metastasis. Accordingly, the degree of vascularization in a tumor has been correlated with metastatic potential and prognosis of the disease. All these critical steps—tumor growth, metastasis, and angiogenesis—involve ECM degradation and correlate with matrix metalloproteinase (MMP) activity. Therefore, 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 target in blocking tumor metastasis and angiogenesis.

Rath and Pauling postulated that natural substances, such as lysine and ascorbic acid (vitamin C) have a potential to control tumor growth and invasion through their critical role in the stability of connective tissue and as natural inhibitors of ECM degradation [4]. Based on Rath's approach, we developed a unique, nontoxic nutrient mixture (NM) that has shown significant antitumor effects in a number of cancer cell lines in vitro and in experimental tumor models in vivo [5–7]. NM has been effective in inhibition of Matrigel invasion of several cancer cell lines, and MMPs 2 and 9 secretion both in vitro and in vivo. It has exhibited strong antiangiogenic properties using models such as the chorioallantoic membrane (CAM) assay in chick embryos and basic fibroblast factor (bFGF)-induced vessel growth in C57BL/6 female mice [8].

OBJECTIVE

Melanoma is one of the neoplasias that most frequently metastasizes, especially in the lung, where it represents a challenge in oncology, because current treatments are ineffective with a high level of toxicity and mortality. Several studies have demonstrated that intravenous injection of melanoma cells into C57BL/6 mice results in pulmonary metastasis, providing an excellent model of metastasis to assess the effectiveness of test agents on tumor cell extravasation and formation of tumors [9–12]. The primary objective of this study was to investigate whether a mixture of natural components (NM) could inhibit experimentally induced lung metastases in C57BL/6 female mice injected with melanoma B16FO cells.

MATERIALS AND METHODS

Materials

The melanoma B16FO cells, media, and sera used were obtained from ATCC (American Type Culture Collection, Rockville, MD), and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY.

Cancer Cell Lines and Culture

Murine melanoma B16FO cells were maintained in Dulbecco's modified Eagle's (DME) medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 48 hours, the cultured cells in each group were detached by trypsinizing, washed with phosphate-buffered saline (PBS), and diluted and suspended to the test concentrations indicated in the specific experiments in 0.2 mL PBS for inoculation. Some cells were cultured for 18 hours with the nutrient mixture at 500 μg/mL; then the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to the concentration of 5×10^4 in 0.2 mL PBS.

Animals

Female C57BL/6 mice, approximately 6 weeks of age on arrival, were purchased from Simonsen Laboratories (Gilroy, CA) and maintained in microisolator 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.

Experimental Design

Experiment 1

After 1 week of isolation, 5- to 6-week-old female C57BL/6 mice ($n = 36$) were divided into 6 groups of 6 mice each: Groups 1 to 4 were injected via tail vein with B16FO melanoma cells (5×10^4/mouse), group 5 was injected in the tail vein with the same number of B16FO melanoma cells pretreated with NM (500 μg/mL for 18 hours), and group 6 (untreated control) was injected with the vehicle saline. Consequently, the mice were placed on the following diet regimens and administration routes for NM: Groups 1 and 5 were fed Purina Chow, the control diet; group 2 mice were fed the same diet supplemented with 0.5% NM (*w*/*w*); group 3 mice were given NM intraperitoneally (IP) (4 mg/mouse, 3 × /week × 2 weeks) and fed the control diet; group 4 was given NM intravenously (IV) (4 mg/mouse, 3 × /week × 2 weeks and fed the control diet. Group 6 mice were fed the control diet. Two weeks later, the mice were sacrificed, and their lungs were excised, weighed, fixed in 10% (*v*/*v*) buffered formalin, and processed for histopathological examination. The weights of the lungs were obtained and the number of metastases and number of cells in each metastatic lesion were counted. After injection of cancer cells, the general condition of each animal was assessed daily. Body weights at onset of study and at termination were recorded.

Experiment 2

After 1 week of isolation, 5- to 6-week-old female C57BL/6 mice (n = 24) were divided into 4 groups (A to D) of 6 mice each, and were injected into the tail vein with either 10^5 or 10^6 B16FO melanoma cells, as follows: Groups A and B were injected with 10^5 melanoma cells/mouse. mouse. Groups C and D were injected with 10^6 melanoma cells/mouse. Groups A and C mice were continued on Purina Chow, the control diet, and Groups B and D were fed the control diet supplemented with 0.5% NM. Two weeks later, the mice were sacrificed, and their lungs were excised, weighed, fixed in 10% (*v*/*v*) buffered formalin, and processed for histopathological examination. The weights of the lungs were obtained and the number of metastases and number of cells in each metastatic lesion were obtained. After injection of cancer cells, the general condition of each animal was assessed daily. Body weights at onset of study and at termination were measured.

Composition of Nutrient Mixture

The nutrient mixture (NM) was composed of the following in the ratio indicated: 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*-acetylcysteine 200 mg; standardized green tea extract 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Lab). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, (-)-epigallocatechin gallate (EGCG) 35%, and caffeine 1.0%); selenium 30 mg; copper 2 mg; manganese 1 mg.

The composition of the nutrient mixture (NM) was designed by defining critical physiological targets in cancer progression and metastasis. These include ECM integrity and control of MMP activity. ECM formation and structure is dependent upon adequate supplies of ascorbic acid and the amino acids lysine and proline, which assure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for optimal collagen and ECM formation. Lysine is a natural inhibitor of plasmin-induced proteolysis and, as such, plays an important role in ECM stability [4, 13]. *N*-acetylcysteine has been observed to inhibit MMP-9 activity [14] and invasive activities of tumor cells [15]. Selenium has been shown to interfere with MMP expression and tumor invasion [16], as well as migration of endothelial cells through ECM [15]. Green tea extract has shown to be a promising agent in controlling angiogenesis and metastasis [17]. In addition to addressing ECM properties, some nutrients are critical in inducing cancer cell death. A recent study confirmed that ascorbic acid inhibits cancer cell division and growth through production of hydrogen peroxide [18]. Because arginine is a precursor of nitric oxide (NO),

any deficiency of arginine can limit the production of NO, which has been shown to play an important role in the induction of apoptosis in breast cancer cells [19].

Based on our previous studies, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetylcysteine, selenium, copper, and manganese would result from their synergy. 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 [20]. Thus by including nutrients such as *N*-acetylcysteine, arginine, selenium, manganese, and copper in addition to ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG.

Statistical Analysis

Data are expressed as means ± SD for the groups. Data was analyzed by independent sample *t* test.

RESULTS

Experiment 1

Effect of NM on Lung Weight of C57BL/6 Mice Injected with 5×10^4 B16FO Melanoma Cells

The mean lung weight (0.16 ± 0.02 g) of the mice injected with melanoma cells was 12.5% ($P = .05$) greater than the mean lung weight (0.14 ± 0.01 g) of the untreated control group. Mice injected with melanoma cells and treated with different protocols did not differ significantly between groups, except for the NM IP group (group 3), which had a mean lung weight (0.13 ± 0.01 g) 19% ($P = .008$) less than the injected control (group 1).

Effect of NM on the Number of Metastatic Colonies and Histopathology of Lungs of C57BL/6 Mice Injected with 5×10^4 B16FO Melanoma Cells

Lungs isolated from mice injected with 5×10^4 B16FO melanoma cells and fed the NM diet (group 2) had fewer metastatic colonies than did the lungs of mice fed the control diet (group 1). Furthermore, pulmonary metastatic colonies were completely absent in the lungs of mice injected with the same number of melanoma cells pretreated with NM (group 5). Figure 1 presents the gross lung photographs of representative groups and Figure 2 presents the average numbers of pulmonary metastatic colonies in all tested groups of mice and the percent inhibition. The average number of isolated colonies in mice fed the NM diet (group 2) was reduced

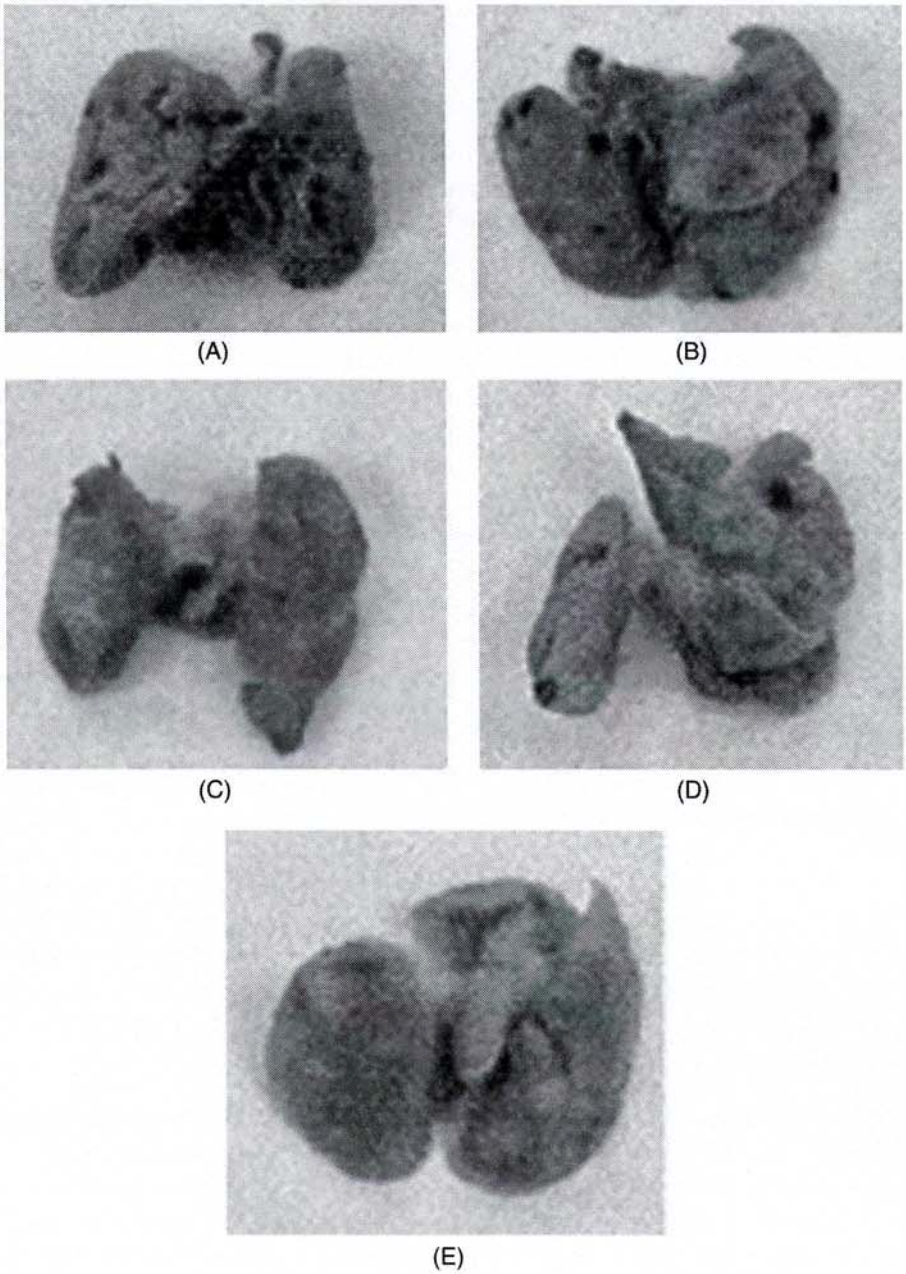

FIGURE 1 Effect of NM on lungs of C57BL/6 mice Injected with B16FO cells (5×10^4): gross lung photographs. (*A*) Group 1 (control diet); (*B*) group 2 (NM diet); (*C*) group 3 (NM IP); (*D*) group 4 (NM IV); (*E*) group 5 (NM pretreated cells).

by 63% ($P < .0001$) compared to the control diet (group 1). Pulmonary colonization was inhibited by 86% ($P < .0001$) in mice receiving NM by

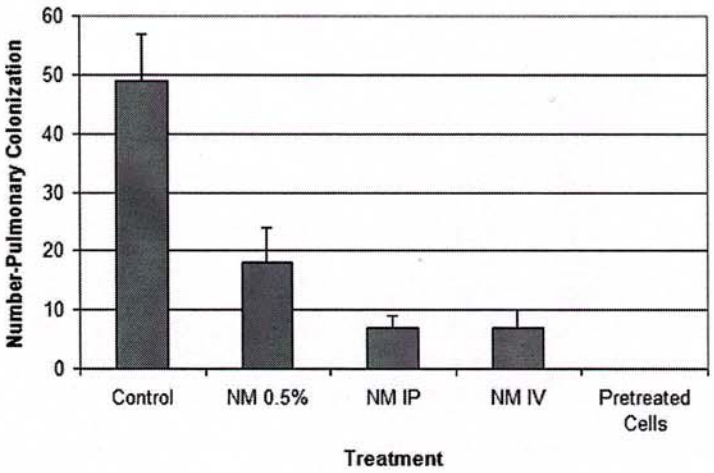

FIGURE 2 Effect of NM on pulmonary colonization of B16FO cells (5×10^4) injected in C57BL/6 mice.

IP and IV injections (groups 3 and 4). The lungs from mice injected with melanoma cells pretreated with NM (group 5) were free (100% blockage, $P < .0001$) from any metastasis. Thus, exposing melanoma cells to NM inhibited their ability to metastasize (the lungs are completely clear), without need for diet supplementation, suggesting profound changes on a cellular level.

Experiment 2

Effect of NM on Mean Lung Weight of C57BL/6 Mice Injected with Different Numbers of B16FO Melanoma Cells

Effect of NM on the weight of lungs in mice 2 weeks post injection with different numbers of melanoma cells is presented in Figure 3. Total lung weight in mice fed the control diet was not significantly different in animals injected with 5×10^4 and 1×10^5 melanoma cells (0.16 ± 0.02 and 0.15 ± 0.03 g, respectively). However, the weight of the lungs from mice injected with 1×10^6 melanoma cells was significantly ($P = .0001$) greater and averaged 0.92 ± 0.11 g. Mice fed NM had lower lung weights than mice fed the control diet. The average lung weight of mice injected with 10^6 melanoma cells and fed the NM diet was 0.56 ± 0.05 versus 0.92 ± 0.11 g in mice on control diets. This difference was statistically significant ($P = .0001$). The mean lung weight in mice injected with 1×10^5 cells and fed NM was 0.11 g, compared to 0.15 g in mice fed the control diet. However, this difference did not reach statistical significance. Lung weights were pooled and compared to number of injected melanoma cells. An excellent correlation was obtained ($r = .93$, $P < .0001$).

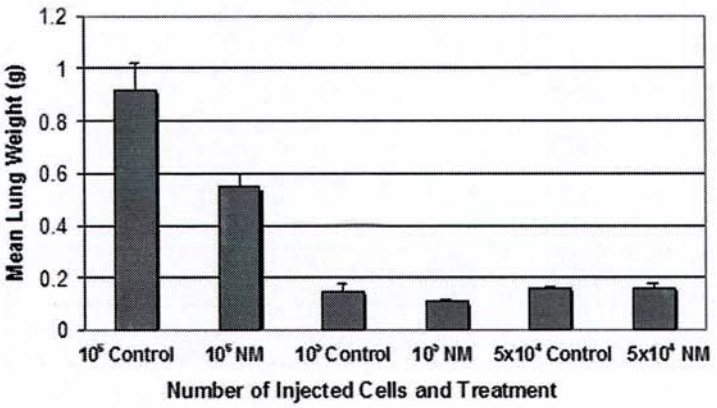

FIGURE 3 Relationship between number of injected melanoma cells and lung weight of C57BL/6 mice.

Effect of NM on Histopathology of Lungs of C57BL/6 Mice Injected with 10^6 and 10^5 B16FO Melanoma Cells

The nutrient mixture also had significant inhibitory effect on pulmonary metastases in mice that were injected with larger numbers of melanoma cells, such as 10^5 and 10^6 per mice, as shown in gross lung photographs in Figure 4. Pulmonary metastases involved 40% to 45% of the lung in mice injected with 10^5 cells (Figure 4*A, B*), with an average number of colonies of 600 (594 ± 53 [SE]) in the control (group A), whereas in NM-fed (group B) the number of colonies was reduced to 400 (408 ± 18 [SE]), a reduction of 33%. After injection with 10^6 melanoma cells, the lungs from the mice fed a control diet (group C) and the nutrient supplement (group D) were full of metastases (black spots occupy almost the entire lung), and thus difficult to count (Figure 4*C, D*). However, the mean weight of lungs mice supplemented with the NM diet post injection with melanoma cells (group D) was significantly less than the lung weight from the control group (group C), as discussed in the previous section.

DISCUSSION

Currently there is no effective treatment to blocking metastasis, and discovery in this area, especially when involving nontoxic approaches, would dramatically improve cancer prognosis. Rath and Pauling postulated [4] that cancer growth and metastasis can be controlled through a natural approach, targeting the stability of the connective tissue surrounding cancer and therefore contributing to tumor "encapsulating" effect. Previous studies [21] demonstrated that NM treatment of fibroblasts increases fibroblast-produced ECM stability by increasing collagen IV to collagen I

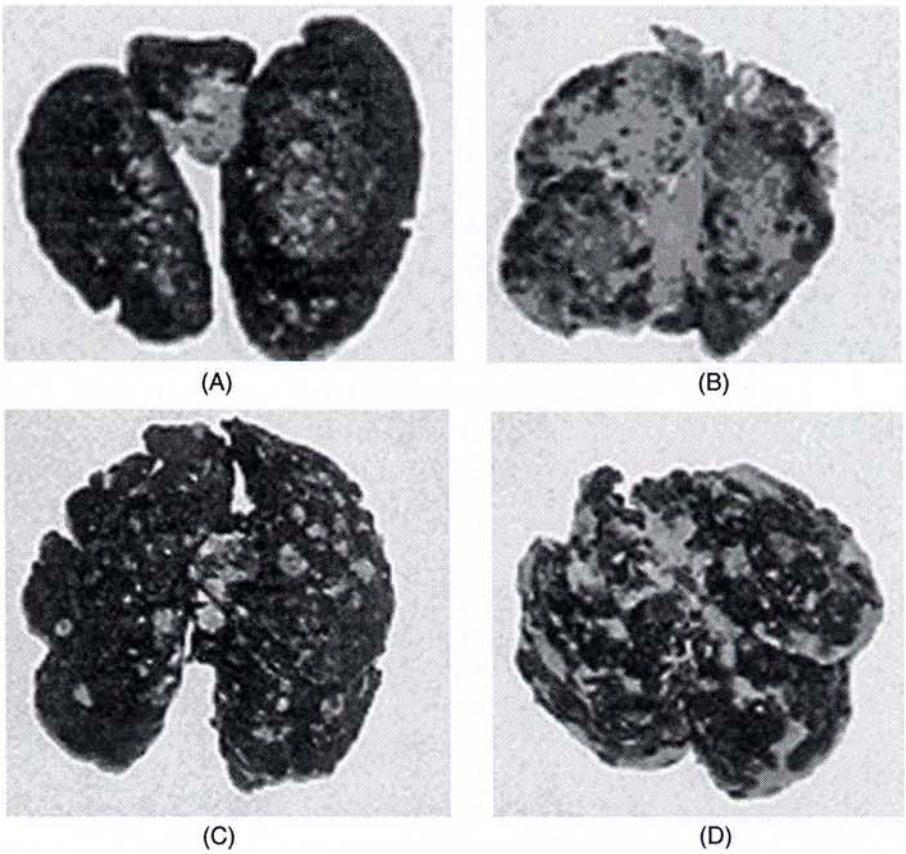

FIGURE 4 Effect of NM on lungs and pulmonary colonization of B16FO cells ($1 \times 10^5$and 1×10^6) injected in C57BL/6 mice: gross lung photographs. Group A and B mice were injected with 10^5 B16FO cells/mouse. Group A mice were continued on a control diet and group B were fed the control diet supplemented with 0.5% NM. Group A (control group) showed metastasis with back-to-back colonies, numbering 600 on average and group B showed metastasis with back-to-back colonies, numbering 400 colonies on average. Groups C and D were injected with 10^6 B16FO cells/mouse. Group C mice were continued on a control diet and group D were fed the control diet supplemented with 0.5% NM. Group C and D mice showed metastasis involving most of the lobe. (*A*) Group A (control diet); (*B*) group B (NM diet); (*C*) group C (control diet); (*D*) group D (NM diet).

ratio. In addition, the fibroblast-produced ECM under different NM concentrations showed inhibitory effects on proliferation of osteosarcoma cells (MNNG-HOS, U2OS, and Ewing's sarcoma SK-ES.1), as well as significantly decreased matrix invasive properties of osteosarcoma cancer cells plated on these matrices. Because this approach targets a common mechanism involved in growth and invasion of all cancer types, it has a potential to be applied in a variety of cancers. This approach is based on key nutrients essential for connective tissue stability: lysine and ascorbic acid. As such, the synthesis and structure of collagen fibrils depends upon hydroxylation of

proline and lysine residues in collagen fibers catalyzed by ascorbic acid. In addition, lysine is the most abundant amino acid in collagen, therefore important to optimal collagen structure. Neither ascorbate nor lysine are produced in the human body and because their insufficient intake is likely, cellular deficiency of these nutrients can compromise optimum structure and function of collagen and the connective tissue. Studies of NM on ECM constituents in vivo are in progress.

Lysine and its analogues interfere with the activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, and consequently affected the plasmin-induced MMP activation cascade [4]. As postulated earlier by Rath and Pauling [4], the availability of lysine has important implications in cancer metastasis and when combined with ascorbate it has a therapeutic potential [4]. This has been confirmed in a recent study investigating the importance of proteolytic degradation of connective tissue on cancer metastasis [22]. The study demonstrated 7-fold reduction in metastasis of transgenic mammary cancer in plasmin deficient mice [22]. Also, Lee and colleagues reported inhibition of experimental metastasis of melanoma B16BL6 cells in C57BL/6 mice by Cambodian *Phellinus linteus* via regulation of urokinase-type plasminogen activator [23]. Furthermore, expression of urokinase-type plasminogen activator inhibitor type I by human prostate carcinoma cells was shown to inhibit angiogenesis and metastasis to lung and liver in an athymic mouse model [24].

Our previous studies have shown that ascorbic acid and lysine combined with other specific nutrients (NM) can completely inhibit invasion of several cancer cell lines through Matrigel, accompanied by the inhibition of MMP secretion by these cells [5, 25, 26]. MMP degradation of basement membranes is key to cancer cell invasion. Research has shown that highly metastatic cancer cells secrete higher amounts of MMPs than do poorly metastatic cells, indicating that the invasive and metastatic abilities of these cancer cells in vitro and in vivo correlate with MMP-9 expression [27]. In addition, the NM was effective in vitro and in vivo in decreasing angiogenesis, the process that involves enzymatic remodeling of ECM.

The present study was undertaken to investigate whether the nutrient mixture (NM) targeting stability of collagen and connective tissue would affect pulmonary metastasis of B16FO melanoma cells in C57/BL6 mice. Thus, our design involved: exposure of naïve melanoma cells to NM in the diet, through IP delivery (bypassing intestinal absorption, and before their injection in mice. The results show that dietary supplementation of mice with NM markedly reduced pulmonary metastases in animals injected with different numbers of melanoma cells (from 5×10^4 to 1×10^6). Interestingly, inhibition of pulmonary metastases was more pronounced in animals receiving NM either by IV or IP delivery (86%), compared to dietary

supplementation (63%). This suggests that exposure to higher nutrient concentrations than those obtained through diet is more effective. These findings corroborate with a recently published National Institutes of Health (NIH) study [28], which demonstrated that vitamin C could selectively kill several cancer cell types when used in high doses, such as provided through IV injections.

Furthermore, our study indicates that exposure of melanoma cells for 18 hours to NM before injecting them into mice completely prevented the formation of metastatic lung tumor nodules, which would indicate a direct effect of NM on melanoma cell metabolism. In current studies (unpublished data), exposure of melanoma B16FO cells to NM resulted in only slight apoptosis (assessed by caspase activity image-iT live green poly caspases detection kit; Molecular Probes Invitrogen Detection Technologies) at 100 μg/mL and moderate at 500 μg/mL concentration. (The results are still being evaluated.) Thus, the B16FO cells cultured for 18 hours with the nutrient mixture at 500 μg/mL prior to injection probably underwent apoptosis as our in vitro data indicates moderate apoptosis at that concentration. This is in accordance with the study conducted on four leukemia cell lines (human T-lymphocytic leukemia virus type 1 [HTLV-1] dependent and independent), which demonstrated that this NM used at nontoxic concentrations was effective in triggering apoptosis and inhibiting cellular proliferation [29].

Thus, preincubation of melanoma cells with NM before injecting them into a tail vein probably affected cell viability at 18 hours. In recent in vitro studies (unpublished data) with melanoma B16FO cells, we did not detect NM toxicity based on MTT assays at 100 μg/mL concentration, but about 50% toxicity over the control was observed at 500 and 1000 μg/mL. Morphological evaluation by hemotoxylin and eosin (H&E) did not indicate any changes up to 1000 μg/mL.

Though our model does not reproduce all steps of metastasis, such as tumor cell separation from the primary tumor and invasion of the vascular system, it does standardize the onset of invasion by injection of tumor cells into tail veins of the mice, and measure the ability of malignant cells to penetrate the vascular basement membrane to exit and penetrate the lung interstium to begin to proliferate and form tumors in the lungs. According to Fidler [30], melanoma cells are cleared from the blood circulation within 24 hours after IV injection, indicating the tumor cells have a brief period of time to reach the lung interstitium and begin cell proliferation and tumor development.

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 antiangiogenic actions on malignant cell lines [31–34]; in addition, low levels

of ascorbic acid have been reported in cancer patients [35–37]. Green tea extract is a potent anticancer agent that has been reported to have antimetastatic and antiangiogenic effects against human cancer cell lines [38–41]. 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 [42]. Though it is obvious that the nutrient mixture is effective in preventing tumor metastasis as a whole, we intend to investigate the synergistic antimetastatic effect of each nutrient such as ascorbic acid, lysine, proline, arginine, and green tea extract in vivo in the near future.

CONCLUSIONS

The results of the present study show that this nutrient mixture was effective in inhibiting metastasis of B16FO melanoma cells in C57BL/6 mice, especially when nutrients were delivered IV or IP. Preincubation of tumor cells with NM completely prevented development of lung tumors in these mice. These findings together with our earlier results clearly indicate anticancer and antimetastatic potential of the NM. Furthermore, use of the nutrient mixture would not pose any toxic effect clinically, especially in the relevant doses, as in vivo safety studies demonstrate. During an in vivo study on possible toxicity from NM, we found that NM had neither adverse effect on vital organs (heart, liver, and kidney), nor on the associated functional serum enzymes [43]. In addition morphology studies of melanoma B16FO cells exposed to different concentrations of NM showed no effect on morphology even at 1000 μg/mL. Taking into account nutrient safety there is an urgent need for confirming effectiveness of this approach in clinical settings.

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