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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

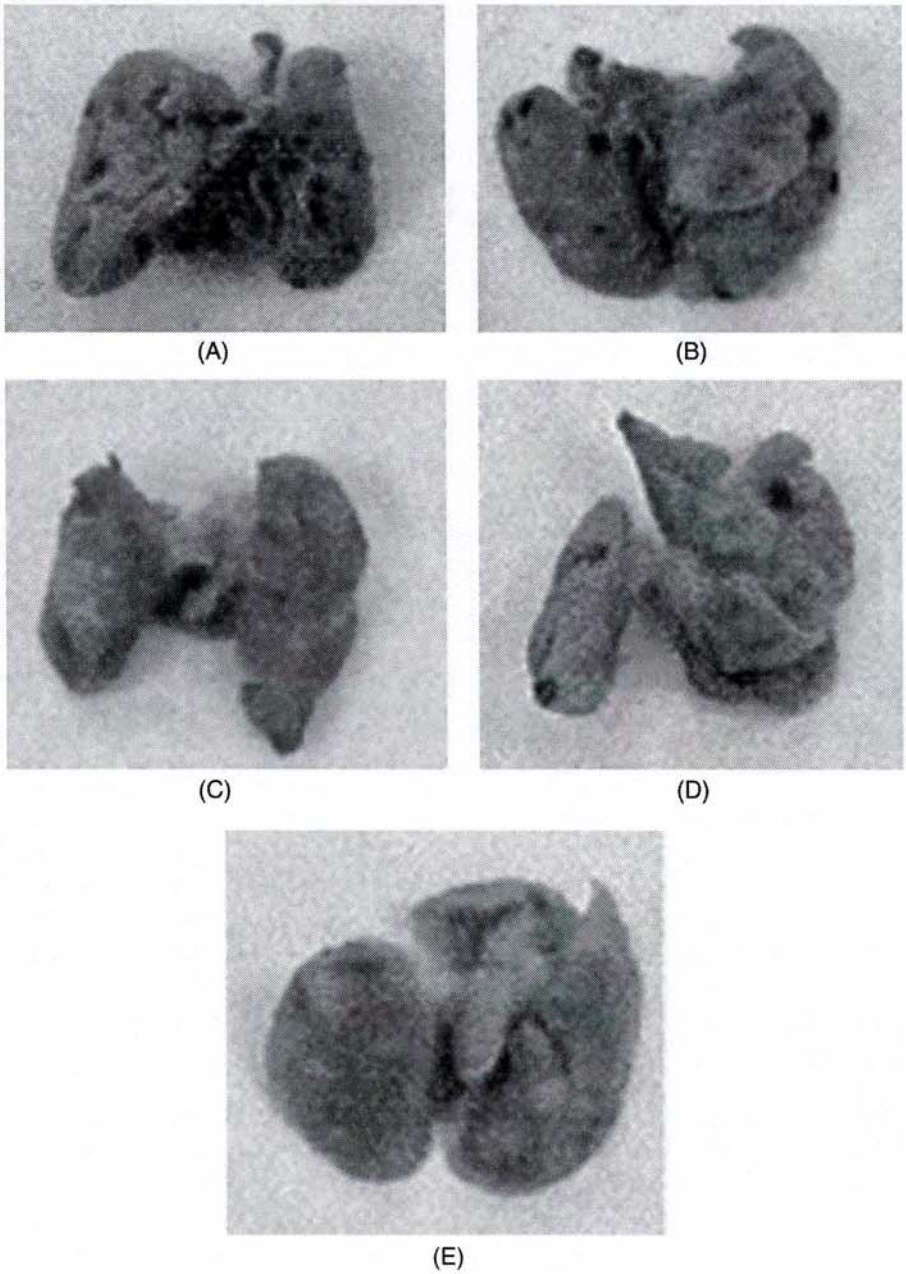

FIGURE 1 Effect of NM on lungs of C57BL/6 mice Injected with B16FO cells (5×10^4): gross lung photographs. (*A*) Group 1 (control diet); (*B*) group 2 (NM diet); (*C*) group 3 (NM IP); (*D*) group 4 (NM IV); (*E*) group 5 (NM pretreated cells).

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

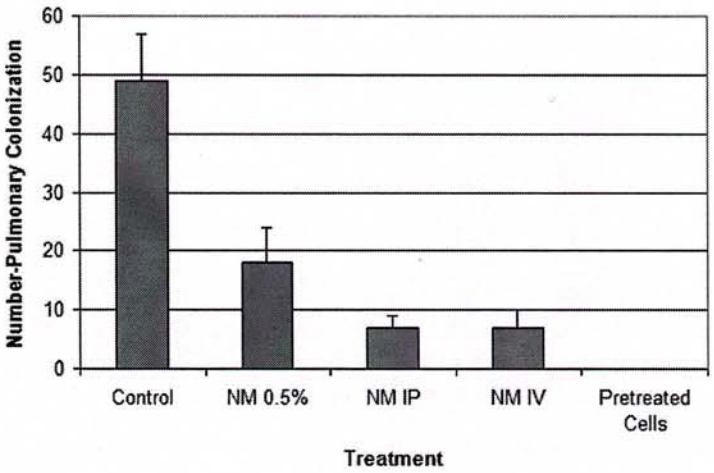

FIGURE 2 Effect of NM on pulmonary colonization of B16FO cells (5×10^4) injected in C57BL/6 mice.

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 ($r = .93$, $P < .0001$).

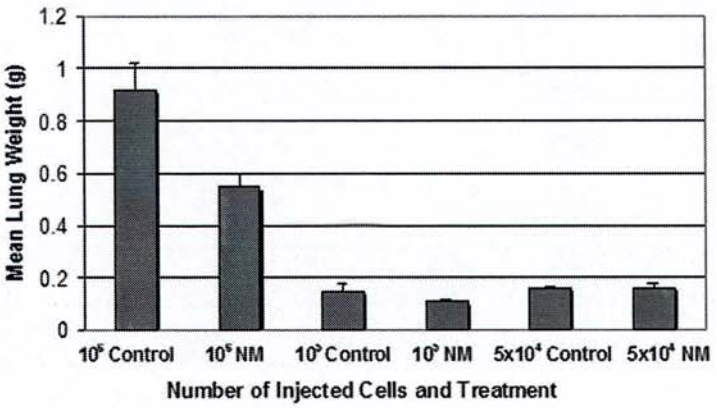

FIGURE 3 Relationship between number of injected melanoma cells and lung weight of C57BL/6 mice.

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

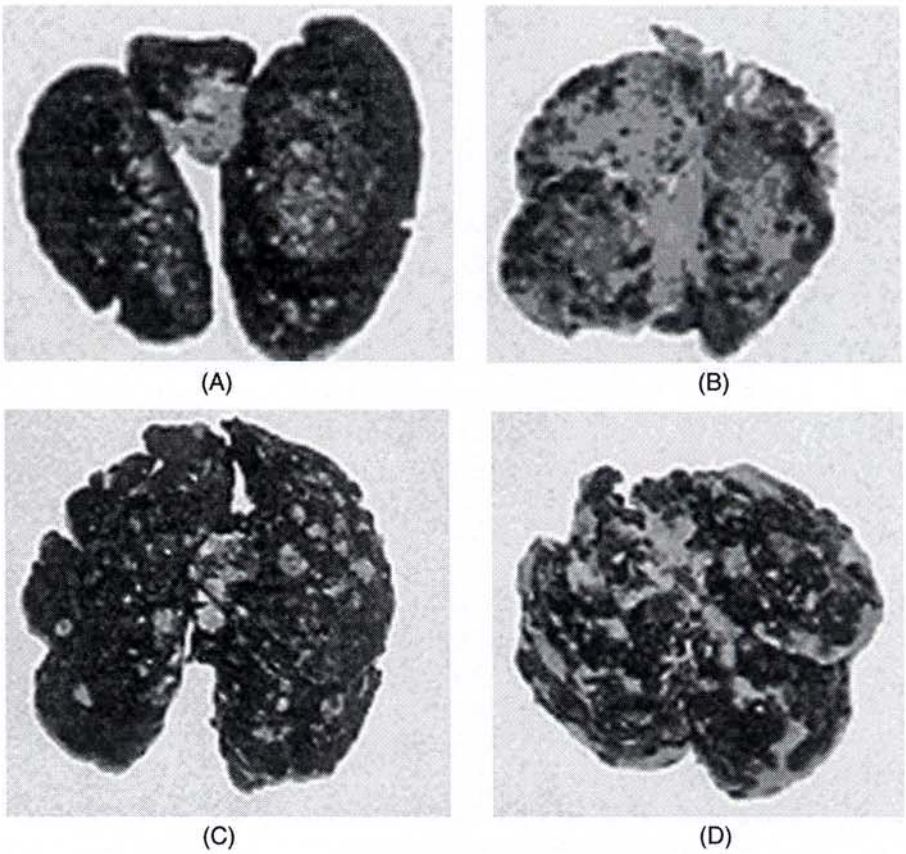

FIGURE 4 Effect of NM on lungs and pulmonary colonization of B16FO cells ($1 \times 10^5$and 1×10^6) injected in C57BL/6 mice: gross lung photographs. Group A and B mice were injected with 10^5 B16FO cells/mouse. Group A mice were continued on a control diet and group B were fed the control diet supplemented 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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