

Suppression of growth and hepatic metastasis of murine B16FO melanoma cells by a novel nutrient mixture

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Abstract. Highly metastatic melanoma is resistant to existing therapies. Our main objective was to investigate the effect of a nutrient mixture (NM) on B16FO tumor growth and hepatic metastasis. Tumor growth was studied in athymic nude male mice, 5-6 weeks old, inoculated with 10^6 B16FO melanoma cells subcutaneously and fed either a regular diet or one supplemented with 0.5% NM. Four weeks later, the mice were sacrificed and their tumors excised, weighed and processed for histology. Metastasis was studied in C57BL/6 mice, which received 10^6 B16FO melanoma cells by intrasplenic injection, as well as a regular or 0.5% NM-supplemented diet for 2 weeks. Survival was studied in C57BL/6 mice receiving 10^6 B16FO melanoma cells intraperitoneally (i.p.) followed by the regular, NM-supplemented, or regular diet in addition to being administered with 2 mg NM injection 3 times per week. NM inhibited the growth of B16FO melanoma cells by 50%. Lesions in the two groups were consistent with malignant melanoma. Mice were injected with B16FO cells in the spleen. Those fed the regular diet developed large black spleens and livers indicating growth in the spleen and metastasis to the liver. In contrast, mice supplemented with NM showed less growth in spleen, but also reduced metastasis to the liver. The survival time of mice receiving NM supplementation and B16FO cells i.p. was greater than in mice which were fed the regular diet. To confirm effects *in vivo*, we investigated the effect of NM on murine B16FO melanoma cells *in vitro*, including cell proliferation by MTT assay, morphology by hematoxylin and eosin (H&E) staining and apoptosis using live green caspase detection kit. *In vitro*, NM was not toxic at 100 $\mu\text{g/ml}$ concentration, but exhibited 44% toxicity over the control at 500 and 1000 $\mu\text{g/ml}$. H&E did not indicate any changes up to 100 $\mu\text{g/ml}$. NM induced slight apoptosis at 100 $\mu\text{g/ml}$, moderate at 500 and extensive at 1000 $\mu\text{g/ml}$ concentration.

Introduction

Melanoma causes the majority of skin cancer-related deaths, due to metastasis to other areas of the body, such as lymph nodes, lungs, liver, brain or bone. Though often curable in its early stages, metastatic malignant melanoma is an extremely aggressive cancer with no current viable treatment (1).

In a previous study we demonstrated that a nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract was effective in inhibiting the pulmonary metastasis of B16FO melanoma cells injected into the tail vein of C57BL/6 mice, especially when the nutrients were delivered intravenously or intraperitoneally (i.p.) (2). Currently, orthotopic models of cancer, rather than ectopic ones, are recommended to create clinically accurate animal models to test the effect of potentially therapeutic agents on metastasis, as different biological behavior has been observed in orthotopic versus ectopic locations as well as differences in drug response (3,4). Based on this, we utilized intrasplenic injection of B16FO cells in C57BL/6 mice to investigate the effect of NM on the prevention of experimental hepatic metastasis.

Extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs) plays a critical role in the formation of tumors and metastasis. Research has shown that highly metastatic melanoma and other cancer cells secrete higher amounts of MMPs than do poorly metastatic cells, demonstrating that the invasive and metastatic abilities of these cancer cells *in vitro* and *in vivo* correlate with MMP expression, especially MMP-9 and -2 (5). NM has demonstrated anticancer activity in a number of human cancer cell lines, inhibiting cancer cell growth, MMP secretion, invasion, metastasis and angiogenesis (6,7). A significant component of the basement membrane is type IV collagen and its disruption has been associated with metastases and poor prognoses in several cancer types. A recent study showed that ECM synthesized by normal fibroblasts treated with NM displayed 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 independent of the immune system function and inhibiting critical steps in cancer metastasis (8).

Our main objective was to study the effect of NM on experimental hepatic metastasis by intrasplenic injection of

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B16FO cells into C57BL/6 mice. We also studied the effect of NM supplementation on the primary tumor growth and longevity of mice challenged with an i.p. injection of B16FO cells. The effects of NM on the subcutaneous B16FO melanoma tumor growth, as well as the *in vitro* analysis of NM on B16FO cell proliferation and induction of apoptosis were also assessed.

Materials and methods

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

Composition of the nutrient mixture. Stock solution of the nutrient mixture (NM) was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and 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 μ g; copper, 2 mg and manganese, 1 mg.

In vitro studies

Cell culture. Murine melanoma B16FO cells obtained from ATCC were grown in DME media, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO₂. At near confluence, the cells were treated with the nutrient mixture, dissolved in media and tested at 0, 10, 50, 100, 500 and 1000 μ g/ml in triplicate at each dose. The plates were then returned to the incubator.

MTT assay. Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good indicator of mitochondrial activity and thus cell viability. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) and 500 μ l MTT (Sigma no. M-2128) 0.5 mg/ml in media was added to each well. After the addition of MTT (0.5 mg/ml) the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD₅₇₀ of the DMSO solution in each well was considered to be proportional to the number of cells. The OD₅₇₀ of the control (treatment without supplement) was considered to be 100%.

Morphology and apoptosis. Morphology of cells cultured for 24 h in test concentrations of NM were evaluated by hematoxylin and eosin (H&E) staining, and observed and

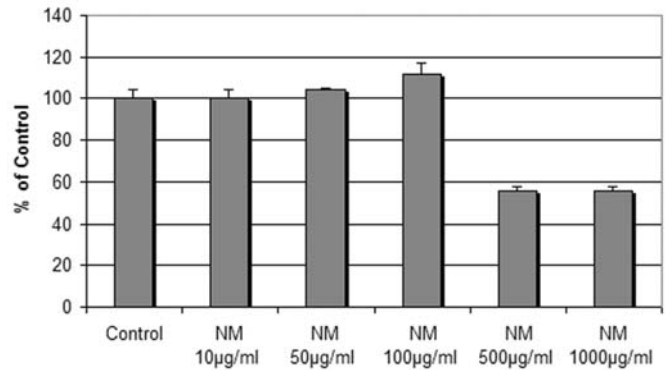


Figure 1. Effect of NM on B16FO melanoma cell proliferation. NM did not exhibit significant antiproliferative effects at 100 μ g/ml, but at 500 and 1000 μ g/ml. The inhibited cell growth by 44% ($p=0.0001$) is shown.

photographed by microscopy. At near confluence, B16FO cells were challenged with NM dissolved in media at 0, 50, 100, 250, 500 and 1000 μ g/ml and incubated for 24 h. The cell culture was washed with PBS and treated with the caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT™ Live Green Poly caspase detection kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represent early and red late apoptosis.

In vivo studies

Tumor growth.

Animals. Male athymic mice (NCR-nu/nu), ~5 weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA and maintained in microisolator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. Animals were cared for according to institutional guidelines for the care and use of experimental animals.

Experimental design. After housing for a week, the mice were inoculated subcutaneously with 10⁶ B16FO melanoma cells (ATCC) in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA). After injection, the mice were randomly divided into: group A which was fed regular Purina mouse chow and group B which was fed the regular diet supplemented with 0.5% NM (w/w). During the study, the mice consumed on average, 4 g of their respective diets per day. Thus, the supplemented mice received ~20 mg of NM per day. After four weeks, the mice were sacrificed and their tumors excised and processed for histology.

Histology. Tissue samples were fixed in 10% buffered formalin. Tissues were embedded in paraffin and cut at 4-5 microns. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with H&E for evaluation using a standard light microscope.

Metastasis from intrasplenic injection of B16FO cells.

Animals. Male C57BL/6 mice, ~5 weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA and maintained in microisolator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. The

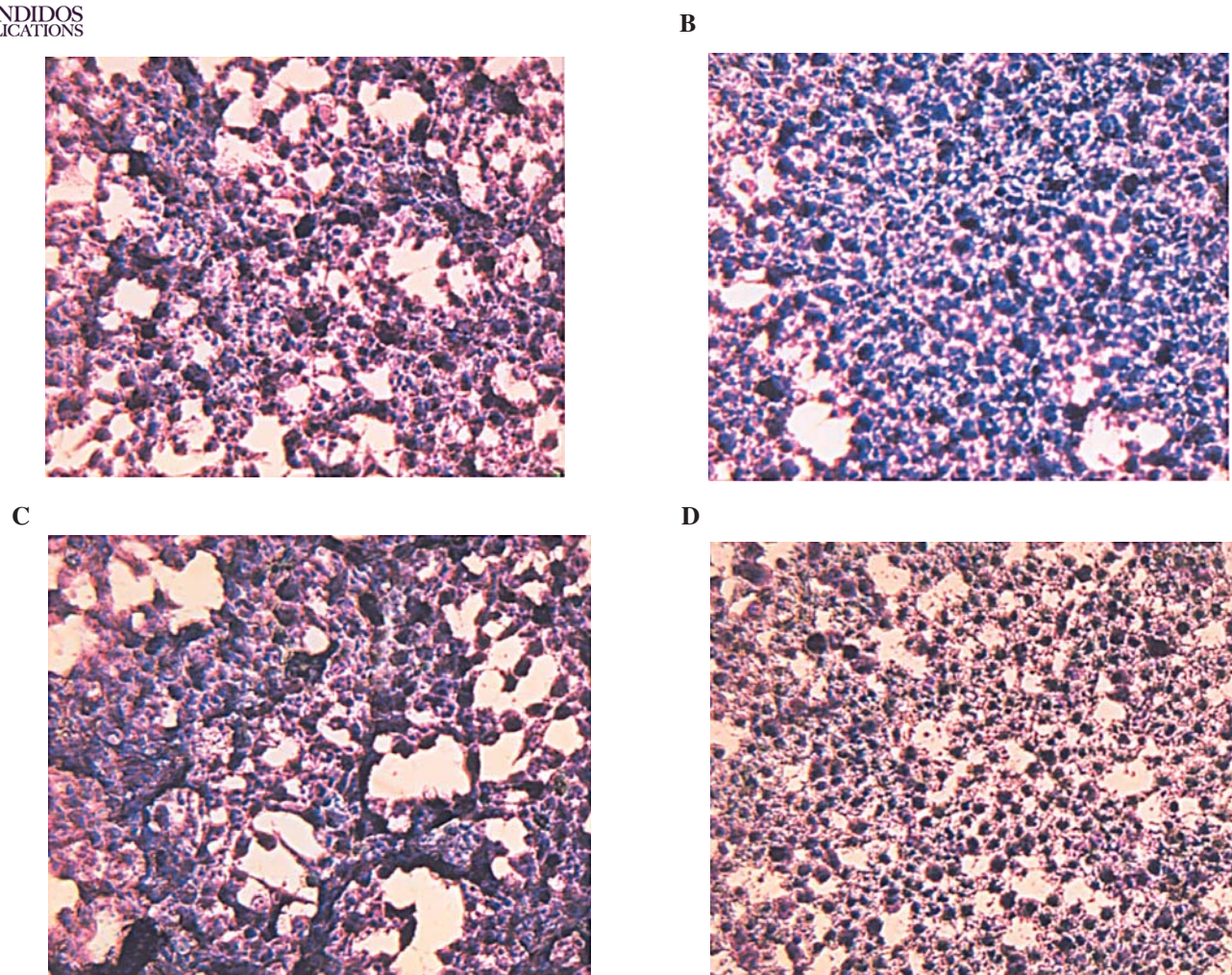


Figure 2. Effect of NM on the morphology of B16FO melanoma cells (H&E). (A) Control, (B) NM 100 $\mu\text{g/ml}$, (C) 500 $\mu\text{g/ml}$ and (D) 1000 $\mu\text{g/ml}$. B16FO melanoma cells exposed to different concentrations of NM did not show any morphological changes at or below 100 $\mu\text{g/ml}$, as shown in Fig. 2A-D. At 1000 $\mu\text{g/ml}$ NM, apoptotic cells, exhibiting shrinkage, condensed and darkly stained nuclei and strong acidophilic cytoplasm, were evident.

animals were cared for according to institutional guidelines for the care and use of experimental animals.

Experimental design. The effect of NM on B16FO cell metastasis from spleen of C57BL/6 male mice was investigated. Mice were anesthetized by isoflourane USP (Abbott Labs, Chicago, IL), hair was shaved on the mid-left side of the abdomen and the area was sterilely prepped. Skin incisions ~1 cm were made in the mid-left abdomen to expose the abdominal cavities of C57BL/6 male mice and spleens were injected with B16FO cells (10^6 in 0.1 ml DME). Cavities were sutured and clamped. Mice were divided into three groups of five mice each. Group 1 received no B16FO cells and regular Purina mouse chow; Group 2, B16FO cells and regular Purina mouse chow and Group 3, B16FO cells and 0.5% NM-supplemented Purina mouse chow. After two weeks, the animals were sacrificed and spleens, livers, kidneys and lungs were excised, examined and weighed. Liver metastasis was evaluated by final liver weight and observation of the melanoma colonies in sectioned livers.

Effect of NM on survival of mice receiving B16FO cells i.p. Eighteen of 24 C57BL/6 male mice were injected with 10^6 murine melanoma B16FO cells i.p. and six were injected

with vehicle. After injection the mice were divided into the following groups of six mice each: Group 1 received no B16FO and regular Purina mouse chow; Group 2, 10^6 B16FO cells i.p. and regular Purina mouse chow; Group 3, 10^6 B16FO cells i.p. and 0.5% NM-supplemented Purina mouse chow and Group 4, 10^6 B16FO cells, regular Purina mouse chow and 2 mg NM injected three times per week. The number of animals surviving was counted daily. At termination of the study, the animals were sacrificed and the intraperitoneal cavity and organs (spleens, livers, kidneys and lungs) were examined.

Results

In vitro studies

Cell proliferation study. NM did not show antiproliferative effects on B16FO cells at 100 $\mu\text{g/ml}$ concentration, but exhibited 44% ($p=0.0001$) toxicity compared to the control at 500 and 1000 $\mu\text{g/ml}$, as shown in Fig. 1.

Morphology and apoptosis. B16FO melanoma cells exposed to different concentrations of NM did not show any morphological changes at or below 100 $\mu\text{g/ml}$, as shown in Fig. 2A-D. At 1000 $\mu\text{g/ml}$ NM, apoptotic cells, exhibiting

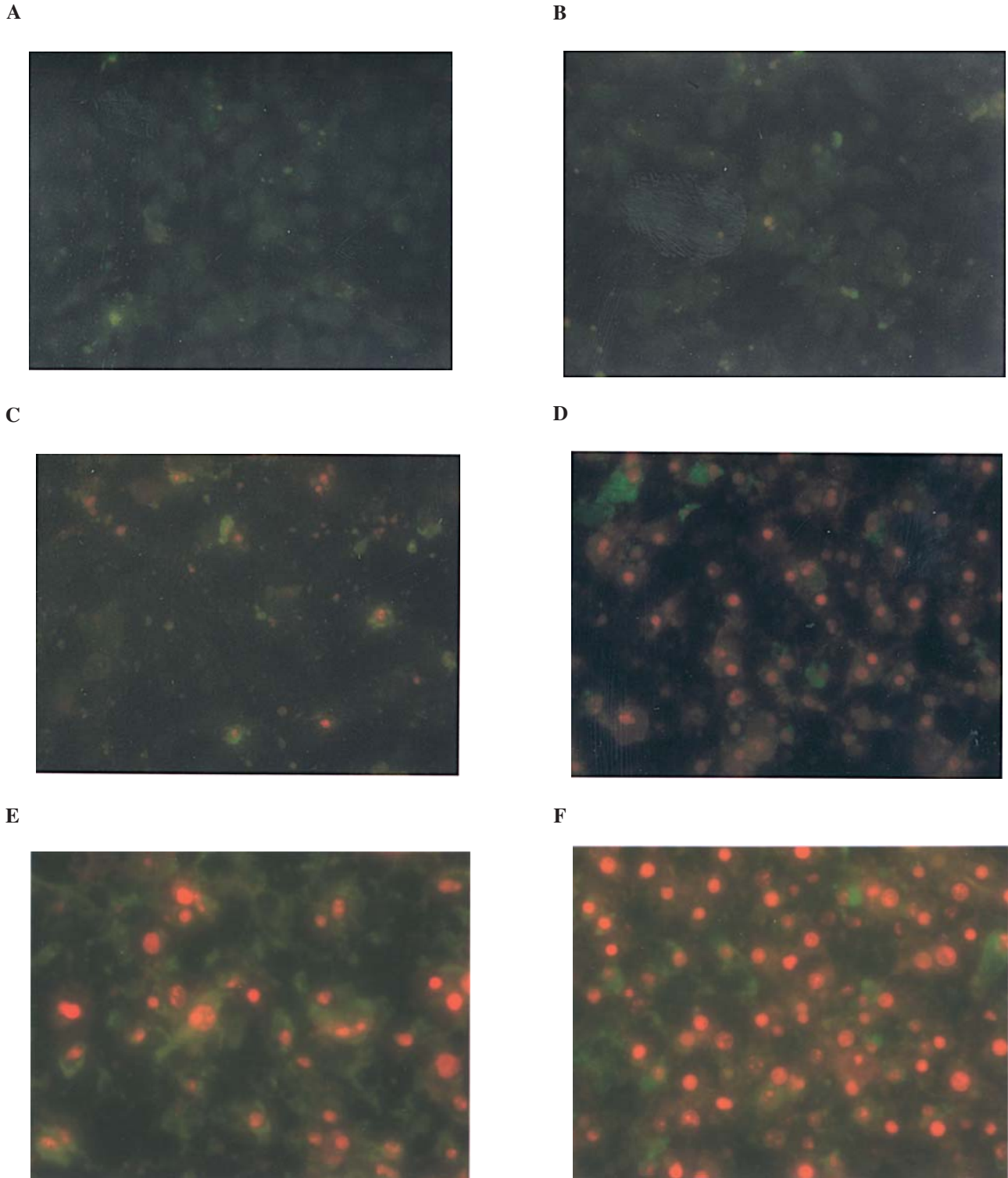


Figure 3. Effect of NM on apoptosis of B16FO cells (Live green caspase detection kit). (A) Control, (B) NM 50 $\mu\text{g/ml}$, (C) 100 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$ and (F) 1000 $\mu\text{g/ml}$. Dose-dependent apoptosis of B16FO cells was evident with the NM challenge.

shrinkage, condensed and darkly stained nuclei and strong acidophilic cytoplasm, were evident. Using the live green caspase kit, dose-dependent apoptosis of B16FO cells was evident with the NM challenge, as shown in Fig. 3A-F. NM induced a slight apoptosis at 100 $\mu\text{g/ml}$ (46% live, 33.3% early apoptotic and 20.6% late apoptotic cells), moderate at 500 $\mu\text{g/ml}$ (15% live, 4.3% early apoptotic and 82.2% late apoptotic cells) and extensive at 1000 $\mu\text{g/ml}$

(9.6% live, 0.2% early apoptotic and 86.2% late apoptotic cells). A quantitative graph of apoptotic cells is shown in Fig. 4.

In vivo studies

Tumor growth. The nutrient-supplemented nude mice developed significantly smaller tumors (reduction in weight by 47%) ($p=0.0002$), as shown in Fig. 5.

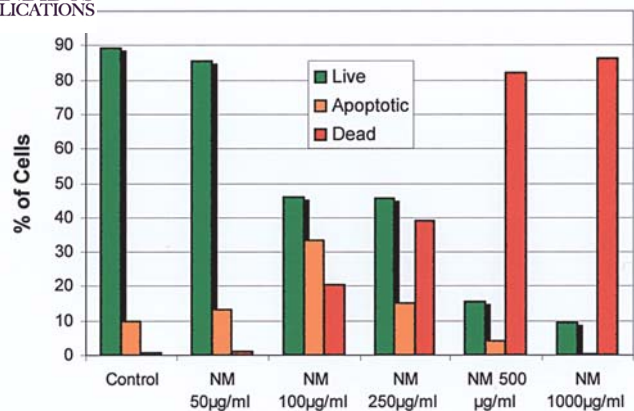


Figure 4. Effect of NM on apoptosis of B16FO cells (Live green caspase detection kit), quantitative analysis. NM induced slight apoptosis at 100 $\mu\text{g/ml}$ (46% live, 33.3% early apoptotic and 20.6% late apoptotic cells), moderate at 500 $\mu\text{g/ml}$ (15% live, 4.3% early apoptotic and 82.2% late apoptotic cells) and extensive at 1000 $\mu\text{g/ml}$ (9.6% live, 0.2% early apoptotic and 86.2% late apoptotic cells).

Histopathology. Lesions in the two groups were composed of cords and nests of large, irregularly round, pigmented cells consistent with a malignant melanoma (Fig. 6A-D). The

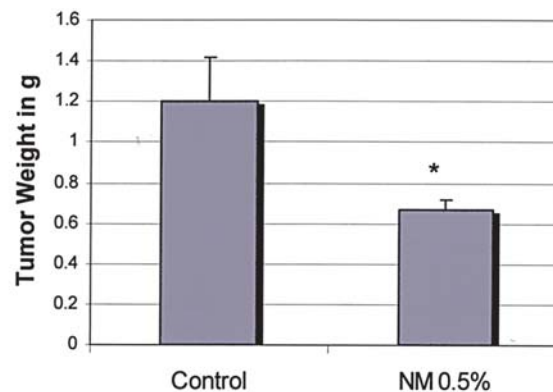
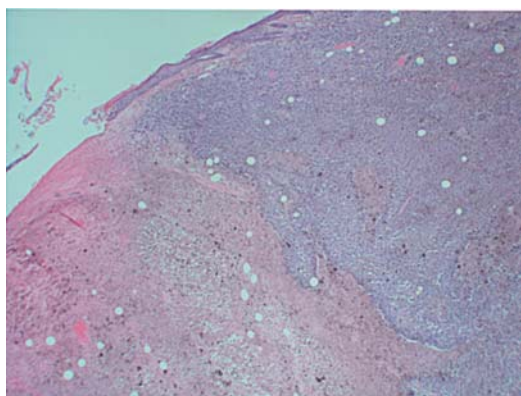


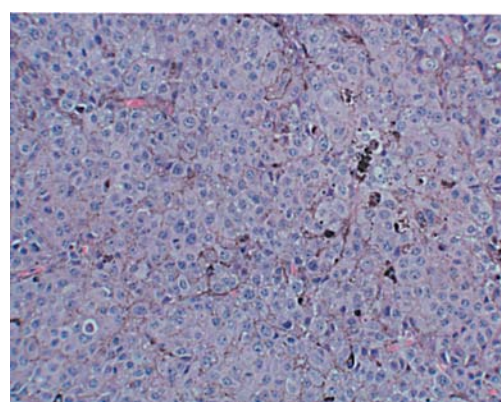
Figure 5. Effect of NM on growth of B16FO melanoma tumors. Supplementation with 0.5% NM reduced the mean tumor size to 53% ($p=0.0002$) of the control mean tumor weight.

control specimen (Fig. 6A and B) consisted of three sections of three irregularly round, expansile and invasive tumors, one of which is ulcerated. Tumor necrosis is present in the ulcerated lesion. The area of necrosis extends from the area of ulceration through the central portion of the tumor involving ~45% of the mass. Foci of necrosis are present in the

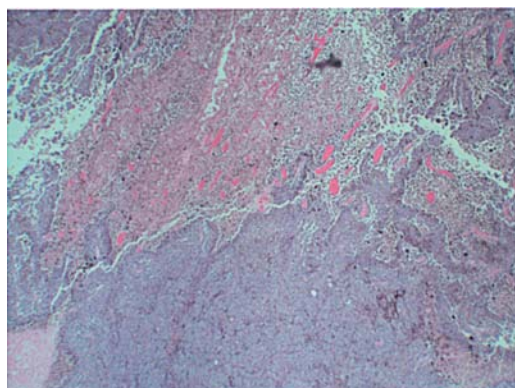
A



B



C



D

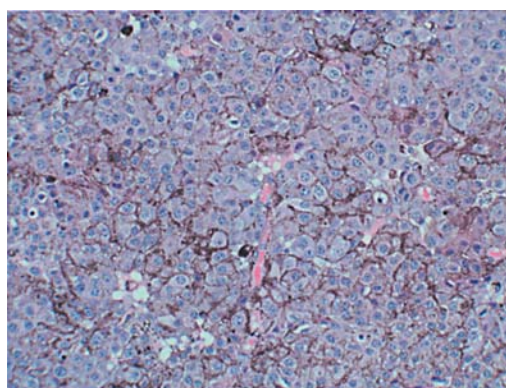


Figure 6. Histopathology of B16FO tumors. Lesions in the two groups were composed of cords and nests of large, irregularly round, pigmented cells consistent with a malignant melanoma. (A) Control, tumor ulceration and necrosis (x40); (B) control, pigmented melanoma cells (x200); (C) 0.5% NM-supplemented, tumor necrosis (x40) and (D) 0.5% NM supplemented-pigmented melanoma cells (x200).

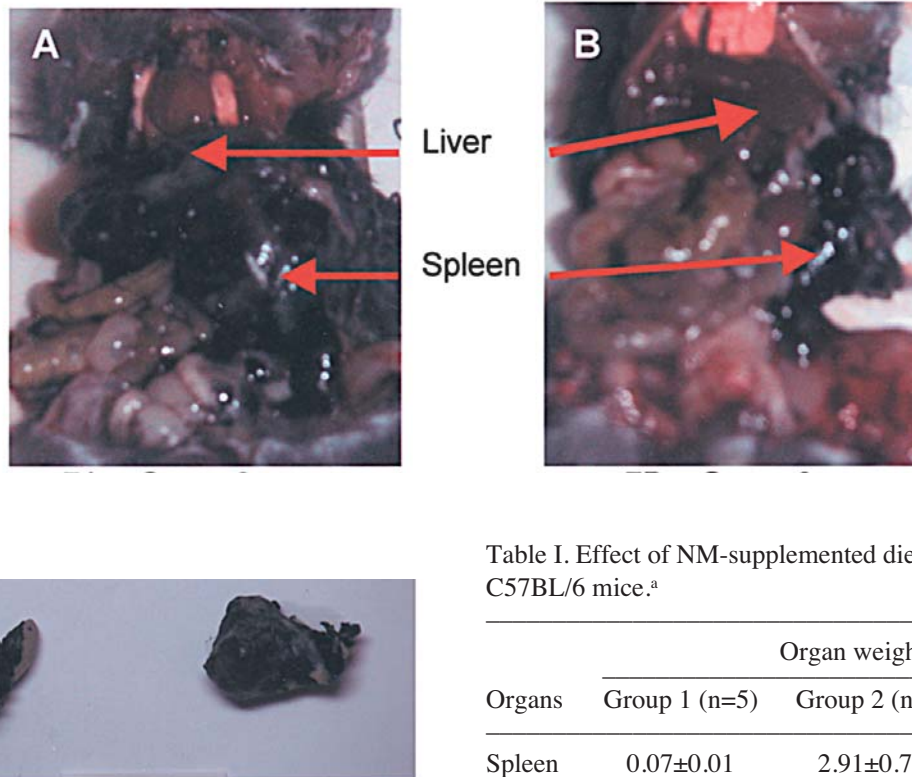


Figure 7. Gross anatomy of representative C57BL/6 mice injected with 10^6 B16FO melanoma cells in the spleen. (A) Group 2 (B16FO cells + regular diet), (B) Group 3 (B16FO cells + 0.5% NM-supplemented diet) and (C) representative spleens from Groups 2 and 3. Mice fed the regular diet (Group 2) developed large black spleens and livers indicating growth in the spleen and metastasis to the liver. However, mice supplemented with NM (Group 3) not only showed less intrasplenic growth of melanoma compared to Group 2 mice, but also significantly less metastasis to the liver. In all groups, no metastasis to the kidneys or lungs was evident as these organs were clear of B16FO cells.

remaining two lesions involving ~10% of these tumors. The three lesions are composed of cords and nests of large, irregularly round, pigmented cells consistent with a malignant melanoma. The specimen from the NM-supplemented mice (Fig. 6C and D) consists of three non-ulcerated subcutaneous tumors. The lesions are somewhat smaller than those noted in the control, but are morphologically similar to the non-ulcerated control lesions.

Metastatic post-intrasplenic injection of B16FO cells in C57BL/6 mice. Of the C57BL/6 mice injected with B16FO cells in the spleen, the mice that were fed the regular diet (Group 2) developed large black spleens and livers indicating growth in the spleen and metastasis to the liver, as shown in Fig. 7A. However, the mice supplemented with NM (Group 3) not only showed less intrasplenic growth of melanoma compared to the control mice, but also significantly less metastasis to the liver (Fig. 7B). In all groups, no metastasis to the kidneys nor lungs was evident as these organs were clear of B16FO cells. Organ weights for the various groups, shown in Table I, support the gross anatomical conclusions. Splenic tumor growth was reduced significantly (36%, $p=0.002$) in the NM-supplemented group compared to the

Table I. Effect of NM-supplemented diet on organ weights of C57BL/6 mice.^a

Organs	Organ weights (g)		
	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)
Spleen	0.07±0.01	2.91±0.70	1.90±0.50
Liver	1.11±0.18	2.45±0.40	1.45±0.14
Kidney	0.30±0.04	0.31±0.01	0.35±0.20
Lung	0.13±0.01	0.21±0.02	0.18±0.01

^aGroup 1, no treatment, regular diet; Group 2, 10^6 B16FO melanoma cells injected in spleen followed by the regular diet; and Group 3, 10^6 B16FO melanoma cells injected in spleen followed by 0.5% NM-supplemented diet. The mean weight of spleens in mice receiving an intrasplenic injection of B16FO cells (Group 2) weighed 42 times ($p<0.0001$) that of the untreated Group 1 mice. NM-supplemented mice receiving the same intrasplenic injection of B16FO cells (Group 3) showed a 36% ($p=0.032$) reduction in mean spleen weight versus Group 2 mice, indicating the inhibition of intrasplenic melanoma growth by NM. Mean liver weight in Group 2 mice was >2 times ($p<0.0001$) greater than in the untreated control (Group 1) mice. NM-supplemented mice receiving the same intrasplenic injection of B16FO cells (Group 3) showed a 41% ($p=0.0007$) reduction in mean liver weight versus Group 2 mice, indicating a significant blockage of hepatic metastasis with NM supplementation.

control.

Hepatic metastasis in NM-supplemented mice was reduced by 41% ($p=0.0007$) compared to the control group, based on the mean liver weights of the groups. To ensure that the organ weight differences were due to tumor growth, the mean weights of the groups were determined, and found not to be significantly different (Group 1, 21.5±2.5 g; Group 2, 21.7±0.5 g and Group 3, 21.3±0.7 g).

Survival of C57BL/6 mice post-i.p. injection of B16FO cells. When C57BL/6 mice received B16FO cells i.p., NM dietary treatment (Group 3) resulted in increased survival over Group 2 mice, as shown in the Kaplan-Meier survival curve in Fig. 9. The longest survival time (22 days) for the groups was reached by a mouse in Group 3 whose survival was five

Organs	Organ weights (g)			
	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	Group 4 (n=6)
Spleen	0.07±0.01	0.08±0.01	0.096±0.03	0.09±0.02
Liver	1.11±0.02	0.90±0.07	0.92±0.02	1.03±0.02
Kidney	0.30±0.04	0.29±0.02	0.25±0.02	0.31±0.05
Lung	0.13±0.01	0.19±0.06	0.16±0.04	0.15±0.02
Heart	0.11±0.01	0.09±0.01	0.09±0.01	0.09±0.01

^aGroup 1, no B16FO cells, regular diet; Group 2, 10^6 B16FO melanoma cells i.p. followed by regular diet; Group 3, 10^6 B16FO melanoma cells i.p. followed by 0.5% NM-supplemented diet; and Group 4, 10^6 B16FO melanoma cells i.p. followed by regular diet and 2 mg NM 3 times per week.

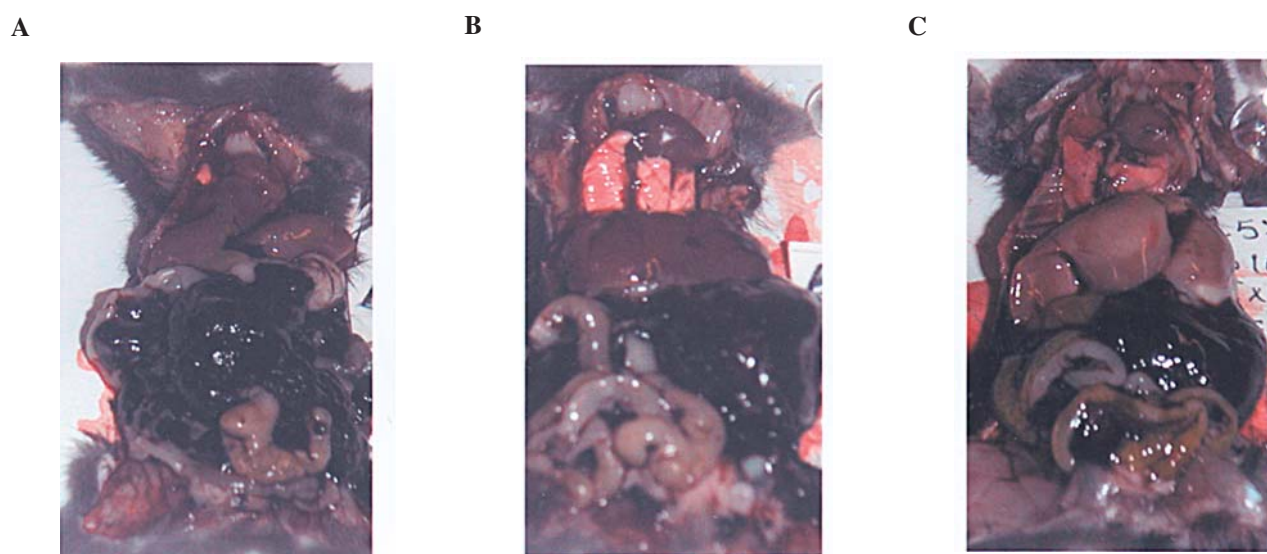


Figure 8. Gross anatomy of representative C57BL/6 mice administered with 10^6 B16FO melanoma cells i.p. (A) Group 2 (B16FO cells + regular diet), (B) Group 3 (B16FO cells + 0.5% NM-supplemented diet) and (C) Group 4 (B16FO cells + regular diet + 2 mg NM three times per week).

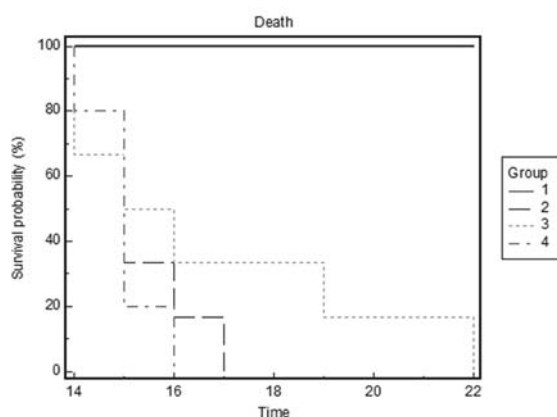


Figure 9. Effect of NM treatment on survival of C57BL/6 mice receiving B16FO melanoma cells i.p. (Kaplan-Meier survival curve). Group 1, no B16FO treatment, regular diet; Group 2, 10^6 B16FO melanoma cells i.p. followed by regular diet; Group 3, 10^6 B16FO melanoma cells i.p. followed by 0.5% NM-supplemented diet and Group 4, 10^6 B16FO melanoma cells i.p. followed by regular diet and 2 mg NM injected 3 times per week. NM dietary treatment increased the mean survival time by 1.5 days over Group 2 mice and a maximum survival time of 22 days (five days longer than the maximum survival time of mice in Group 3).

days longer than that of Group 2 mice. Group 3 mice exhibited a mean increase in survival time of 1.5 days over that of Group 2 mice, which showed a mean survival time of 15.2 days versus 16.7 days for Group 3 mice. Group 4 mice had a mean survival time of 15.2 days, after receiving NM by injection. The B16FO cells grew in the cavity but no metastasis to spleen, liver, kidney, lung, nor heart was evident as these organs were clear of B16FO cells (Fig. 8A-C). Organ weights for the various groups did not differ significantly, as shown in Table II, supporting the gross anatomical conclusions. To ensure the organ weight differences were due to tumor growth, the mean weights of the groups were determined, and found not to be significantly different (Group 1, 21.5 ± 2.5 g; Group 2, 18.9 ± 1.8 g; Group 3, 19.2 ± 2.7 g and Group 4, 21.9 ± 1.7 g) (Fig. 9).

Discussion

In order to obtain clinically accurate models, especially with regard to metastasis, orthotopic models of cancer are suggested

for screening new therapeutic agents based on the premise that the growth environment influences cancer behavior and that representative animal models should recreate disease at particular organs of interest (3,4). In this study, our aim was to analyze melanoma in the environment of the liver and present a model of the disease. Our model is identical to that used in the metastatic studies of pancreatic cancer (9) and thyroid carcinoma (10) and is designed to represent the hematogeneous dissemination of melanoma malignancy. In our study, supplementation with the nutrient mixture suppressed B16FO melanoma cell hepatic metastasis after intrasplenic injection in C57BL/6 mice. In this experimental metastasis model, the intrasplenic injection of B16FO cells was used to induce the cells into portal circulation, and allow the tumor cells to form liver metastases. The mice developed metastases in the liver indicating that the model was highly reproducible, carried a high metastatic rate, and was therefore a preferable model for experimental liver metastasis. Findings revealed treatment with NM not only reduced intrasplenic melanoma growth by 36%, but also blocked hepatic metastasis by 41%. The intraperitoneal injection of B16FO melanoma cells into C57BL/6 mice in the control and NM-supplemented groups demonstrated intraperitoneal growth and ascites, but did not result in metastasis to other organs. Longevity, however, increased with NM supplementation compared to the control, but the difference did not reach statistical significance.

Concerning tumor growth, we demonstrated that supplementation with dietary nutrient mixture significantly suppressed murine melanoma B16FO tumor growth in immune-impaired (athymic) mice. The *in vitro* studies supported these findings as they demonstrated a significant inhibition of cell proliferation at 500 $\mu\text{g/ml}$ NM as well as a strong induction of apoptosis at 500 $\mu\text{g/ml}$, suggesting that the inhibition of tumor growth was due probably to the induction of apoptosis. These findings are in agreement with our previous ones in that exposure of melanoma cells for 18 h to NM prior to injecting the cells in mice completely prevented the formation of metastatic lung tumor modules (2).

The nutrient mixture (NM) was designed by defining critical physiological targets in cancer progression and metastasis, such as ECM integrity and MMP activity. ECM formation and structure depends on 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 is a natural inhibitor of plasmin-induced proteolysis and, as such plays an important role in ECM stability (11,12). The green tea extract has shown to be a promising agent in controlling cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (13-17). N-acetyl cysteine has been observed to inhibit MMP-9 activity (18) and invasive activities of tumor cells (19). Selenium has been shown to interfere with MMP secretion and tumor invasion (20), as well as migration of endothelial cells through ECM (19). In addition to addressing ECM properties, some nutrients are critical in inducing cancer cell death. A previous study confirmed that ascorbic acid inhibits cell division and growth through the production of hydrogen peroxide (21). 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, such as in breast cancer cells (22).

In conclusion, the results of the present study show that the nutrient mixture was effective in inhibiting melanoma B16FO cells *in vitro* and tumor growth in nude mice, and that the nutrient mixture had significant inhibitory effects on melanoma B16FO intrasplenic growth and hepatic metastasis in C57BL/6 mice. These findings together with our earlier results clearly indicate the anticancer potential of 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 effects on vital organs (heart, liver and kidney), nor on the associated functional serum enzymes (Roomi MW, *et al.*, J AM Coll Nutr 22: abs. no. 86, 477, 2003).

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