

## ANTIANGIOGENIC PROPERTIES OF A NUTRIENT MIXTURE IN A MODEL OF HEMANGIOMA

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The pathogenesis of hemangiomas is still largely unknown and the current therapy, such as systemic corticosteroid, vincristine, and interferon-alpha, is toxic and remains unsatisfactory. A nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract has shown significant anti-angiogenic and anti-tumor effect against a number of cancer cell lines. *Aim:* Using a mouse hemangioendothelioma model, we investigated the efficacy of NM. We also tested the effect of NM *in vitro*, evaluating cell viability, MMP secretion, invasion, morphology and apoptosis. *Methods:* Athymic nude mice, 5–6 weeks old, were inoculated with  $3 \times 10^6$  EOMA cells subcutaneously and randomly divided into two groups; group A was fed a regular diet and group B — a regular diet supplemented with 0.5% NM. Four weeks later, the mice were sacrificed and their tumors were excised, weighed and processed for histology. We also tested the effect of NM *in vitro*. *Results:* NM inhibited the growth of tumors by 50%. *In vitro*, NM exhibited dose response cytotoxicity with 10%, 30% and 55% at 10, 100 and 1000  $\mu\text{g}/\text{ml}$ . Invasion through Matrigel was inhibited at 50, 100 and 500  $\mu\text{g}/\text{ml}$  by 25%, 30% and 100% respectively. NM induced dose-dependent apoptosis of EOMA cells. *Conclusions:* These results suggest that NM may have therapeutic potential in treating infantile hemangioendotheliomas and, perhaps, other cutaneous vascular tumors.

**Key Words:** hemangioma, hemangioendothelioma, nutrients, tumor growth, athymic nude mice, Matrigel invasion, cytotoxicity.

Hemangiomas, the most common vascular tumors in Caucasian infants, occur in approximately 1% of normal neonates [1] and 12% of normal year-old children [2]; the incidence increases to 20% in premature infants weighing less than 1000 g [3]. In a recent study, Dr Drolet *et al.* [4] reported an increased rate of hemangiomas (an increase of 40% in the last 20 years) in the United States and found the increase to be linked to the rise in the frequency of low birth weight infants. These lesions are characterized by rapid proliferation of capillaries during the first year of life (proliferative stage), followed by a slowed growth and regression of the tumor over the next 5–6 years (involuting stage), with complete regression of the lesion in 90% of affected individuals by the age of 10–12 years [1, 5]. Though the majority have minor vascular birthmarks that resolve without treatment, 10% of hemangiomas cause severe skin distortion or problems with vision or breathing [6]. Such hemangiomas are generally treated with agents, such as systemic corticosteroid, vincristine, and interferon-alpha, which are associated with toxic effects. In addition, when these tumors obstruct the airway or deform the cornea, surgical removal becomes necessary.

Although the pathomechanism of hemangioma is unknown, the rapid proliferation of endothelial cells suggests the importance of angiogenesis. Hemangiomas originate from a single endothelial cell precursor and tumors are composed of microvessels lined by mitotically active endothelial cells and pericytes [7]. Proliferating hemangiomas are highly angiogenic with urinary basic fibroblast growth factor

levels 25–50 times the level seen in normal controls [6]. In studying the cellular markers during the phases of hemangioma, Takahashi *et al.* [6] found that the proliferating phase was defined by high expression of type IV collagenases and vascular endothelial growth factor (VEGF) and the involuting phase — by elevated expression of tissue inhibitor of metalloproteinases (TIMP)1. High expression of bFGF and urokinases were seen in both the proliferating and involuting phases. These results may be used to evaluate therapeutic agents [6].

Consumption of a plant-based diet has been associated with prevention of the development and progression of cancer [8, 9]. Antiangiogenic properties of edible plant products, such as flavonoids, have been reported [10, 11]. In previous studies we found that a NM containing lysine, proline, arginine, ascorbic acid, and green tea extract demonstrated significant antiangiogenic activity utilizing the chorioallantoic membrane (CAM) assay in chick embryos and bFGF-induced vessel growth in C57BL/6J female mice in the mouse Matrigel plug assay [12]. Furthermore, *in vitro* we observed that NM decreased osteosarcoma U2OS cell expression of VEGF, angiopoietin-2, bFGF, PDGF and TGF beta-1 [12]. In a study on human umbilical vein endothelial cells (HUVEC), we found NM inhibited HUVEC migration, MMP expression, Matrigel invasion and capillary tube formation [13].

In this study we investigated the effect of an NM-supplemented diet on tumor growth of murine hemangioendothelioma (EOMA) cells administered subcutaneously in male athymic mice. We chose a mouse EOMA model as this model has been utilized to test the efficacy of antiangiogenic medications in inhibiting vascular tumor proliferation [14]. Further, we tested the effect of NM on EOMA cells *in vitro*, including cell proliferation, MMP secretion, Matrigel invasion and apoptosis.

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**Abbreviations used:** CAM – chorioallantoic membrane; EOMA – hemangioendothelioma; HUVEC – human umbilical vein endothelial cells; NM – nutrient mixture.

## MATERIALS AND METHODS

**In vivo studies.** Male athymic mice (NCR-nu/nu), approximately five 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. All procedures were performed according to humane and customary care and use of experimental animals and followed a protocol approved by internal institutional animal safety review committee.

After housing for a week, the mice ( $n = 12$ ) were inoculated subcutaneously with  $3 \times 10^6$  EOMA cells (ATCC) in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA). After injection, the mice were randomly divided into two groups; group A mice were fed regular Purina mouse chow and group B — the regular diet supplemented with 0.5% NM (w/w). During the study, the mice consumed, on the average, 4 g of their respective diets per day. Thus, the supplemented mice received approximately 20 mg of NM per day. After four weeks, the mice were sacrificed and their tumors were excised and processed for histology. Mean weight of mice at initiation of study and termination of study did not differ significantly between the groups.

Tissue samples were fixed in 10% buffered formalin. All 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 hematoxylin and eosin (H & E) for evaluation using a standard light microscope.

**Cell line and culture.** EOMA cell line was obtained from ATCC (American Type Culture Collection, Rockville, MD). EOMA cells were maintained in DME (Dulbecco's modified Eagle's) medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in 24-well tissue culture plates (Costar, Cambridge, MA). The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY.

At near confluence, the cells were treated with the NM, dissolved in media and tested at 0, 10, 50, 100, 500, and 1000  $\mu$ g/ml in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA), 200 ng/ml was added to cells to induce MMP-9 secretion. The plates were then returned to the incubator.

**Composition of the NM.** The 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-acetyl cysteine 200 mg; standardized green tea extract (derived from green tea leaves, was obtained from US Pharma Lab; the certificate of analysis indicated the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35%, and caffeine 1.0%); 1000 mg; selenium 30  $\mu$ g; copper 2 mg; manganese 1 mg.

**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 tetra-

zolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500  $\mu$ l of MTT (Sigma, USA) 0.5 mg/ml in media was added to each well. After MTT addition (0.5 mg/ml) the plates were covered and returned to the 37 °C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD<sub>570</sub> of the DMSO solution in each well was considered to be proportional to the number of cells. The OD<sub>570</sub> of the control (treatment without supplement) was considered 100%.

**Gelatinase zymography.** Gelatinase zymography was performed in 10% Novex Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20  $\mu$ L) were mixed with sample buffer and loaded for SDS-PAGE with Tris glycine SDS buffer, as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37 °C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl<sub>2</sub> at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

**Matrigel invasion.** Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, EOMA cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO<sub>2</sub> for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

**Apoptosis.** At near confluence, EOMA cells were challenged with NM dissolved in media at 0, 100, 500, and 1000  $\mu$ 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 Caspases Detection Kit 135104, Invitrogen). The cells were

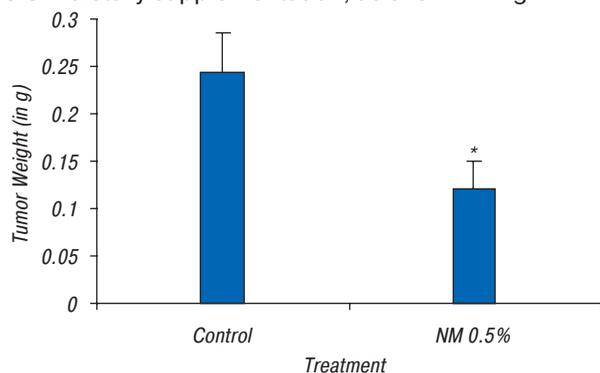
photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represents early apoptosis and red, late apoptosis.

**Statistical analysis.** The results were expressed as means  $\pm$  SD, as indicated in the results, for the groups. Data was analyzed by independent sample «t» test. Pearson's correlation coefficients were determined for toxicity and invasion correlations to NM concentration using MedCalc Software (Markakerke, Belgium).

## RESULTS

### *In vivo*

**Tumor growth.** NM strongly inhibited the growth of EOMA xenografts in nude mice. Mean tumor weight was significantly inhibited (by 50%,  $p = 0.0001$ ) with NM 0.5% dietary supplementation, as shown in Fig. 1.



**Fig. 1.** Effect of NM on growth of EOMA xenografts in male nude mice. NM inhibited the growth of tumors by 50% ( $p = 0.0001$ ). \*Indicates significance of  $p = 0.0001$  with respect to control.

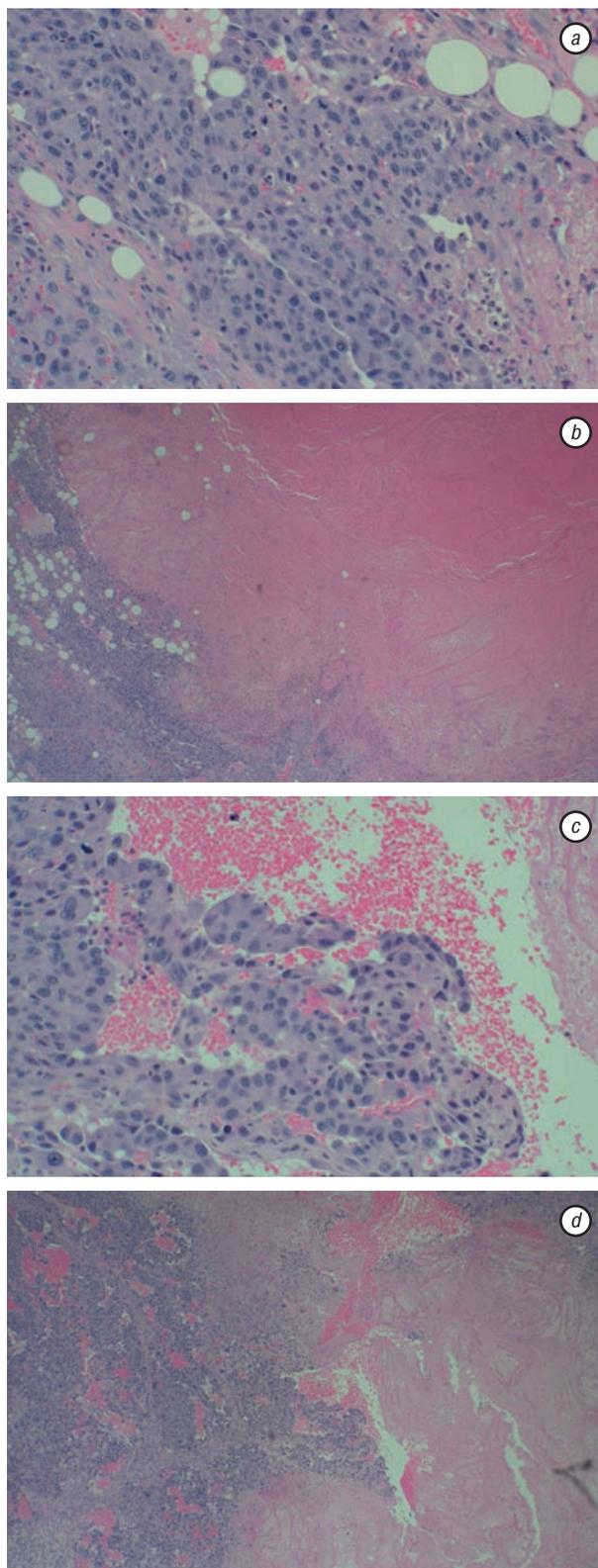
**Histology.** Histologically, the tumors from both groups were round, highly vascular invasive tumors consistent with cavernous hemangioma with thrombosis. Specimens consisted of two sections from similar morphologically round highly vascular invasive tumors composed of large, blood-filled cavitory spaces, lined by highly anaplastic endothelial cells, which in many locations were multilayered. Mitotic figures were infrequent. Fibrin thrombi and associated mild neutrophilic infiltration were common in the cavernous spaces. Tissue from control group showed more extensive thrombosis with associated necrosis and inflammatory cell infiltration than did the supplemented group specimen (Fig. 2, a–d).

### *In vitro*

**Cytotoxicity.** NM exhibited dose response toxicity on EOMA cells *in vitro*, with maximum toxicity of 57% ( $p < 0.0001$ ) over the control at 1000  $\mu\text{g/ml}$ , as shown in Fig. 3. There was significant negative correlation between NM concentration and cell viability, with coefficient  $r = -0.8323$  ( $p < 0.0001$ ).

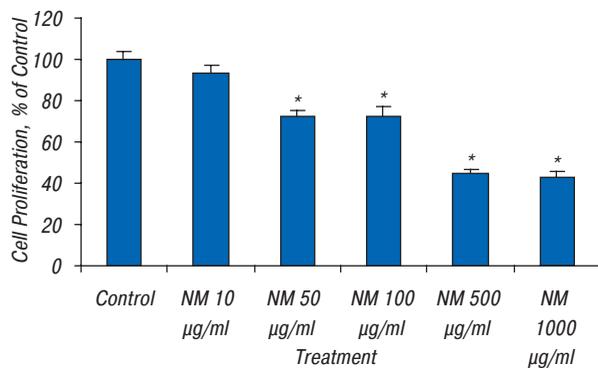
**Gelatinase zymography.** Zymography showed no MMP-2 or MMP-9 secretion by normal or PMA-treated EOMA cells (not shown).

**Matrigel invasion.** NM significantly inhibited EOMA invasion through Matrigel in a dose-dependent manner, with 28% ( $p = 0.0006$ ) inhibition at 100  $\mu\text{g/ml}$  and 100% ( $p < 0.0001$ ) at 500  $\mu\text{g/ml}$ , as shown in Fig. 4 and 5. There was significant negative correlation between NM

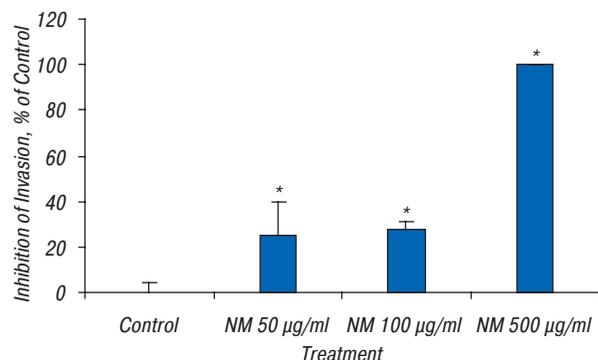


**Fig. 2.** Histopathology of tumors. Histologically, tumor specimens from both groups were consistent with cavernous hemangioma with thrombosis. Specimens consisted of two sections from similar morphologically round highly vascular invasive tumors composed of large, blood-filled cavitory spaces, lined by highly anaplastic endothelial cells, which in many locations were multilayered. Mitotic figures were infrequent. Fibrin thrombi and associated mild neutrophilic infiltration were common in the cavernous spaces. Tissue from control group showed more extensive thrombosis with associated necrosis and inflammatory cell infiltration than did the supplemented group specimen. a, control 40x; b, control 200x; c, NM-supplemented 40x; d, NM-supplemented 200x

concentration and number of EOMA cells that invaded/migrated through Matrigel:  $r = -0.8984$ ,  $p < 0.0001$ .



**Fig. 3.** Effect of NM on viability of EOMA cells. NM exhibited dose response toxicity on EOMA cells *in vitro*, with maximum toxicity of 57% ( $p < 0.001$ ) over the control at 1000 µg/ml. There was significant negative correlation between NM concentration and cell viability, with coefficient  $r = -0.8323$  ( $p < 0.0001$ ). \*Indicates significance of at least  $p = 0.002$  with respect to control.

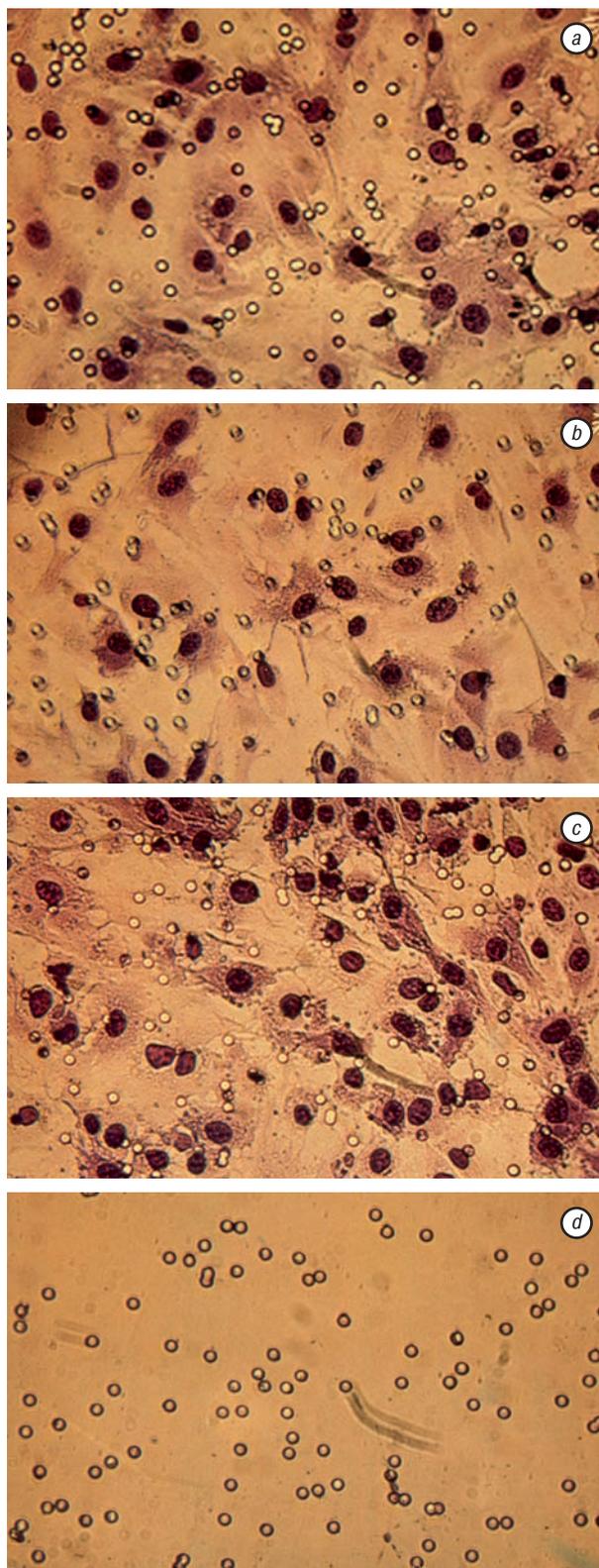


**Fig. 4.** Effect of NM on Matrigel invasion of EOMA cells. NM significantly inhibited EOMA invasion through Matrigel in a dose-dependent manner, with 28% ( $p = 0.0006$ ) inhibition at 100 µg/ml and 100% ( $p < 0.00001$ ) at 500 µg/ml. There was significant negative correlation between NM concentration and number of EOMA cells that invaded/migrated through Matrigel:  $r = -0.8984$ ,  $p < 0.0001$ . \*Indicates significance of at least  $p = 0.05$  with respect to control.

**Apoptosis.** Using the live green caspase kit, dose-dependent apoptosis of EOMA cells was evident with NM challenge, as shown in Fig. 6, a–d. Approximately 50% of cells exposed to 100 µg/ml NM were apoptotic; the number of apoptotic cells increased significantly with increased NM concentration. Quantitative analysis of live, early and late apoptotic cells is shown in Fig. 7. At 100 µg/ml NM, 49% of cells were viable, 16% in early apoptosis and 35% in late apoptosis and at 500 µg/ml NM 2% of cells were viable, 9% in early apoptosis, and 89% in late apoptosis. Virtually all cells exposed to 1000 µg/ml NM were in late apoptosis: 1% viable, 7% in early apoptosis and 92% in late apoptosis.

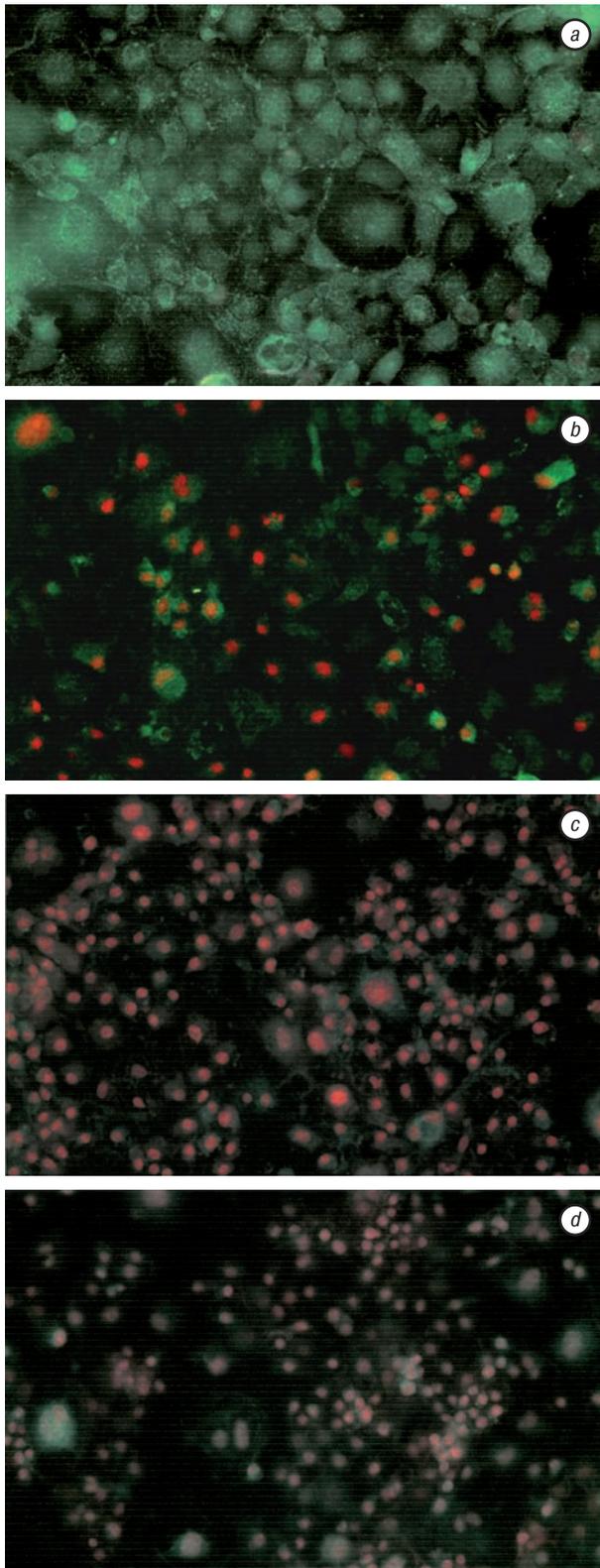
**DISCUSSION**

Using the EOMA mouse model, dietary supplementation with 0.5% NM resulted in a dramatic reduction (57%) in tumor growth in immune impaired (athymic) male nude mice after subcutaneous administration of  $3 \times 10^6$  EOMA cells. Results from the cellular proliferation and apoptosis studies support the *in vivo* findings, as NM showed dose-dependent toxicity in EOMA cells and induced apoptosis in a dose-dependent manner,



**Fig. 5.** Effect of NM on Matrigel invasion: photomicrographs. a, control; b, NM 50 µg/ml; c, NM 100 µg/ml; d, NM 500 µg/ml

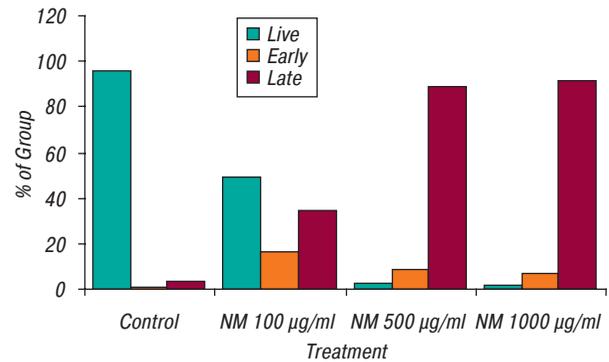
with 55% inhibition of cell growth and apoptotic induction of virtually all cells exposed to 500 µg/ml NM. Thus, mechanisms of NM-induced inhibition of mouse EOMA growth involved both inhibition of proliferation and increased tumor cell apoptosis, as seen in the natural involution of the more common vascular tumor hemangiomas of infancy [15].



**Fig. 6.** Effect of NM on apoptosis of EOMA cells: live-green caspases. Using the live green caspase kit, dose-dependent apoptosis of EOMA cells was evident with NM challenge. Approximately 50% of cells exposed to 100 µg/ml NM were apoptotic; virtually all cells exposed to 500 µg/ml NM were in late apoptosis. a, control; b, NM 100 µg/ml; c, NM 500 µg/ml; d, NM 1000 µg/ml

Invasion of EOMA through Matrigel was also found to be significantly inhibited by NM *in vitro*. As mentioned previously, the proliferating phase of hemangioma is associated with high expression of type IV collagenases

and vascular endothelial growth factor (VEGF) [6]. Degradation of the ECM by migrating endothelial cells and their subsequent invasion of the underlying stroma of neighboring tissues where they organize into new capillary structures are critical in angiogenesis. Regulation of angiogenesis is achieved through a balance of pro- and anti-angiogenic stimuli. The two major pro-angiogenic factors are MMPs that degrade the ECM and vascular endothelial growth factor, a stimulatory factor for cell migration. In our previous study on HUVEC, NM demonstrated a dose-dependent inhibition of capillary tube formation on Matrigel, with completed disruption of tubules at 500 µg/ml [13]. Capillary tube formation in the basement membrane like matrix of Matrigel is a complex process requiring cell-matrix interactions, intercellular communications and cell motility.



**Fig. 7.** Quantitative analysis of live, early and late apoptotic cells. At 100 µg/ml NM, 49% of cells were viable, 16% in early apoptosis and 35% in late apoptosis and at 500 µg/ml NM 2% of cells were viable, 9% in early apoptosis, and 89% in late apoptosis. At 1000 µg/ml NM: 1% viable, 7% in early apoptosis and 92% in late apoptosis

The NM was formulated by selecting nutrients that act on critical physiological targets in cancer progression and metastasis. Adequate supplies of ascorbic acid and the amino acids lysine and proline, are essential for optimal ECM formation and structure as these nutrients insure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine also contributes to ECM stability as a natural inhibitor of plasmin-induced proteolysis [16, 17]. Green tea extract has been shown to control cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression [18–24]. N-acetyl cysteine and selenium have been observed to inhibit MMP-9 and invasive activities of tumor cells, as well as migration of endothelial cells through ECM [25–29]. Ascorbic acid has been shown to inhibit tumor growth via antiangiogenic activity [30] and to inhibit cell division and growth *in vitro* through production of hydrogen peroxide [31]. Arginine is a precursor of nitric oxide (NO); any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells [32].

## CONCLUSIONS

Current therapy for hemangiomas, such as corticosteroids, interferon- $\alpha$  and vincristine, all require systemic administration and are associated with toxic side effects. Thus, they are only used in severe cases

of hemangiomas and most cases are left untreated. The results of the present study show that supplementation with NM was effective in inhibiting growth of EOMA xenografts in nude mice and in inhibiting growth and Matrigel invasion, as well as inducing apoptosis in EOMA cell culture. Furthermore, in contrast to the toxic side effects of current treatments, the NM has been shown to be a safe therapeutic agent. In a previous *in vivo* study addressing safety issues, we found that gavaging adult female ODS rats (weighing 250–300 g) with the NM (at 30, 90 or 150 mg per day for 7 days), had neither adverse effects on vital organs (heart, liver and kidney), nor on the associated functional serum enzymes, indicating that this mixture is safe to use even at these high doses, which far exceed the normal equivalent dosage of the nutrient [33]. While clinical studies are necessary to better determine the efficacy of nutrient therapy in hemangiomas, the results of this study suggest the NM may have therapeutic potential in treating infantile hemangioendotheliomas and other cutaneous vascular tumors with minimal toxic effect.

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