

IN VIVO AND IN VITRO EFFECT OF A NUTRIENT MIXTURE ON HUMAN HEPATOCARCINOMA CELL LINE SK-HEP-1

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Long-term survival of patients with hepatocellular carcinoma (HCC), a common cancer worldwide, remains poor, due to metastasis and recurrence. **Aim:** To investigate the effect of a novel nutrient mixture (NM) containing ascorbic acid, lysine, proline, and green tea extract on human HCC cell line Sk-Hep-1 *in vivo* and *in vitro*. **Methods:** After one week of isolation, 5–6 week old male athymic nude mice were inoculated with 3×10^6 SK-Hep-1 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* on SK-Hep-1 cells, measuring cell proliferation by MTT assay, invasion through Matrigel, apoptosis by green caspase detection kit, MMP secretion by zymography, and morphology by H&E staining. **Results:** NM inhibited tumor weight and burden of SK-Hep-1 xenografts by 42% and 33% respectively. *In vitro*, NM exhibited 33% toxicity over the control at 500 and 1000 $\mu\text{g/ml}$ concentration. Zymography demonstrated MMP-2 and MMP-9 secretion which was inhibited by NM in a dose dependent fashion, with virtual total inhibition at 1000 $\mu\text{g/ml}$. Invasion through Matrigel was inhibited at 100, 500 and 1000 $\mu\text{g/ml}$ by 53%, 83% and 100% respectively. NM induced slight apoptosis at 100 $\mu\text{g/ml}$, and profound apoptosis at 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ concentration. **Conclusions:** These results suggest that NM has therapeutic potential in treatment of HCC. **Key Words:** hepatocellular carcinoma, SK-Hep-1, nutrient mixture, athymic nude mice, apoptosis, invasion.

The prognosis of hepatocellular carcinoma (HCC), which is diagnosed in over 500,000 people worldwide annually, remains dismal despite advances in its clinical study [1, 2]. HCC is much more common in countries in sub-Saharan Africa and Southeast Asia than in the United States, ranking among the most common type of cancer in these regions, and is more common in men than in women [1]. In the U.S., liver and intrahepatic bile duct cancers incidence has been stable and results in 4% of cancer deaths in men and 2% of cancer deaths in women [2]. Average age at diagnosis for liver cancer is 64 and over 90% of people diagnosed with liver cancer are older than 45 years of age [1]. Hepatitis B and C, abuse of alcohol, and exposure to aflatoxin are the most important etiological factors implicated in HCC.

The most prevalent causes of death in patients with HCC include uncontrolled metastasis and recurrence. In recent years, efforts have been focused on exploring many molecular markers related to invasion, metastasis, recurrence and survival in HCC: DNA ploidy, the proliferating activity of tumor cells, tumor suppressor and promoter genes, cell cycle controllers, proteinases that degrade extracellular matrix, adhesion molecules, angiogenic factors and metabolic genes [3]. Among these factors, the matrix metalloproteinases (MMPs) and the plasminogen activation system play crucial roles in cancer invasion and metastasis. Levels of MMP expression were found correlated to recurrence and reduced survival after HCC resection [4, 5].

MMPs are a family of zinc and calcium dependent proteolytic enzymes, are able to degrade connective tissue, among other substrates, such as basement membrane collagen, and have been associated with cancer metastasis and tumor angiogenesis. The gelatinases, especially MMP-9 (gelatinase B) and MMP-2 (gelatinase A), play a key role in degradation of collagen type IV, a main component of the extracellular matrix (ECM). These gelatinases are expressed in hepatocellular carcinoma cells and are associated with progression and invasion of these tumors [4, 6–9]. For example, Guo *et al.* [9] noted positive correlation of MMP-9, MMP-2 and vascular endothelial growth factor (VEGF) expression with recurrence of HCC.

Rath and Pauling [10] postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to modulate tumor growth and expansion. These nutrients can exercise their anti-tumor effect through the inhibition of MMPs and by strengthening connective tissue surrounding cancer cells by influencing collagen synthesis. These two processes are essential for a tumor encapsulating effect. We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid and green tea extract (NM). This nutrient mixture has exhibited synergistic anticancer activity *in vivo* and *in vitro* in a number of cancer cell lines through inhibition of cancer cell growth, MMP secretion, invasion, metastasis, and angiogenesis [11–13]. The present study examines the effect of NM on human HCC cell line SK-Hep-1, *in vivo*, in athymic nude mice bearing SK-Hep-1 xenografts, and *in vitro*, evaluating viability, MMP secretion, invasion and induction of apoptosis.

We designed NM by defining critical physiological targets in cancer progression and metastasis, such as ECM integrity and MMP activity. ECM formation

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Abbreviations used: ECM – extracellular matrix; HCC – hepatocellular carcinoma; H&E – hematoxylin and eosin; NM – nutrient mixture; MMP – matrix metalloproteinase; VEGF – vascular endothelial growth factor.

and structure is dependent upon adequate supplies of ascorbic acid and the amino acids lysine and proline, which insure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine, a natural inhibitor of plasmin-induced proteolysis, plays an important role in ECM stability [10, 14]. Green tea extract has been shown to modulate cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression [15–19]. N-acetyl cysteine has been observed to inhibit MMP-9 activity [20] and invasive activities of tumor cells [21]. Selenium has been shown to interfere with MMP secretion and tumor invasion [22], as well as migration of endothelial cells through ECM [21]. In addition to addressing ECM properties, some nutrients are critical in inducing cancer cell death. A recent study confirmed that ascorbic acid inhibits cell division and growth through production of hydrogen peroxide [23]. Since arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells [24].

MATERIALS AND METHODS

Cancer cell line and culture. Human hepatoma cell line SK-Hep-1 was obtained from ATCC (American Type Culture Collection, Rockville, MD). SK-Hep-1 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY.

Composition of the 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-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 µg; copper 2 mg; manganese 1 mg.

IN VIVO STUDIES

Animals. 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-hour light/12-hour 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.

Experimental design. After housing for a week, the mice (n = 12) were inoculated subcutaneously with 3×10^6 SK-Hep-1 cells 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. Dimensions (length and width) of tumors were measured using a digital caliper, and the tumor burden was calculated using the following formula: $0.5 \times \text{length} \times \text{width}$. Mean weight of mice at initiation of study and termination of study did not differ significantly between the groups.

Histology. 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.

IN VITRO STUDIES

Cell culture. Human SK-Hep-1 cells were grown in MEM, 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 µg/ml in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA), 100 ng/ml was added to cells to induce MMP-9 secretion. 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 index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma #M-2128) 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 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 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 mL) 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 CaCl₂ 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, SK-Hep-1 cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO₂ for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

Morphology and apoptosis. Morphology of cells cultured for 24 h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy. At near confluence, SK-Hep-1 cells were challenged with NM dissolved in media at 0, 100, 500, and 1000 µg/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 ± 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 STUDIES

Tumor growth and incidence. NM strongly inhibited the growth and incidence of SK-Hep-1 xenografts in nude mice. Mean tumor weight was inhibited by 42% ($p = 0.09$) with NM 0.5% dietary supplementation, as shown in Fig. 1, a and tumor burden was inhibited by 36% ($p = 0.005$), as shown in Fig. 1, b. Tumor incidence decreased 33% with NM supplementation; all mice in

the control group developed tumors, while only 4 of 6 mice did on the supplemented diet. Representative gross tumors from both groups are shown in Fig. 2.

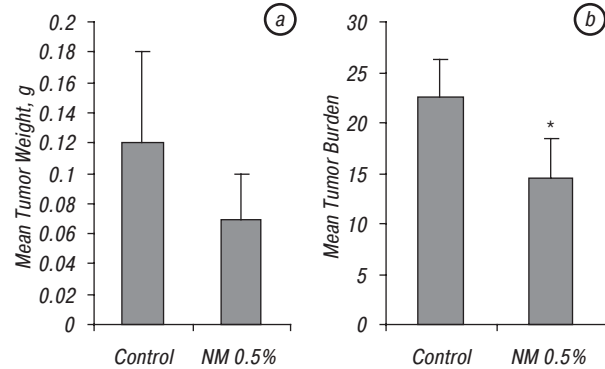


Fig. 1. a, effect of NM on mean tumor weight of SK-Hep-1 xenografts in male nude mice inoculated subcutaneously with 3×10^6 SK-Hep-1 cells. Each group was composed of 6 animals. b, effect of NM on tumor burden of SK-Hep-1 xenografts in male nude mice inoculated subcutaneously with 3×10^6 SK-Hep-1 cells. Each group was composed of 6 animals. *Indicates significance of $p = 0.005$ with respect to control.

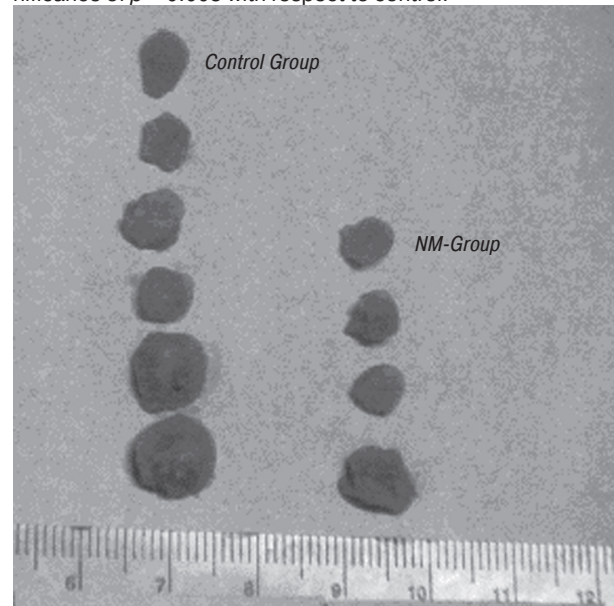


Fig. 2. Gross tumors from male nude mice inoculated subcutaneously with 3×10^6 SK-Hep-1 cells. Each group was composed of 6 animals. All mice in the control group developed tumors while only 4 of the 6 mice in the NM-supplemented group developed tumors

Histology. Histologically, the tumors from both groups were composed of solid nests of irregularly round to oval epithelioid cells with large irregularly round nuclei and cytoplasm with indistinct borders. Stromal spindle-shaped cells that were intermixed with inflammatory cells, predominantly macrophages, surrounded tumor nests, as well as the tumor masses. Focal areas of necrosis involved about 30% of the tumor tissue (Fig. 3, a–d).

IN VITRO STUDIES

Cytotoxicity. NM exhibited no toxicity to SK-Hep-1 cells *in vitro* at lower concentrations, but 33% ($p = 0.002$) toxicity over control at 500 and 1000 µg/ml, as shown in Fig. 4. There was significant negative correlation between NM concentration and cell viability, with coefficient $r = -0.8622$ ($p < 0.0001$).

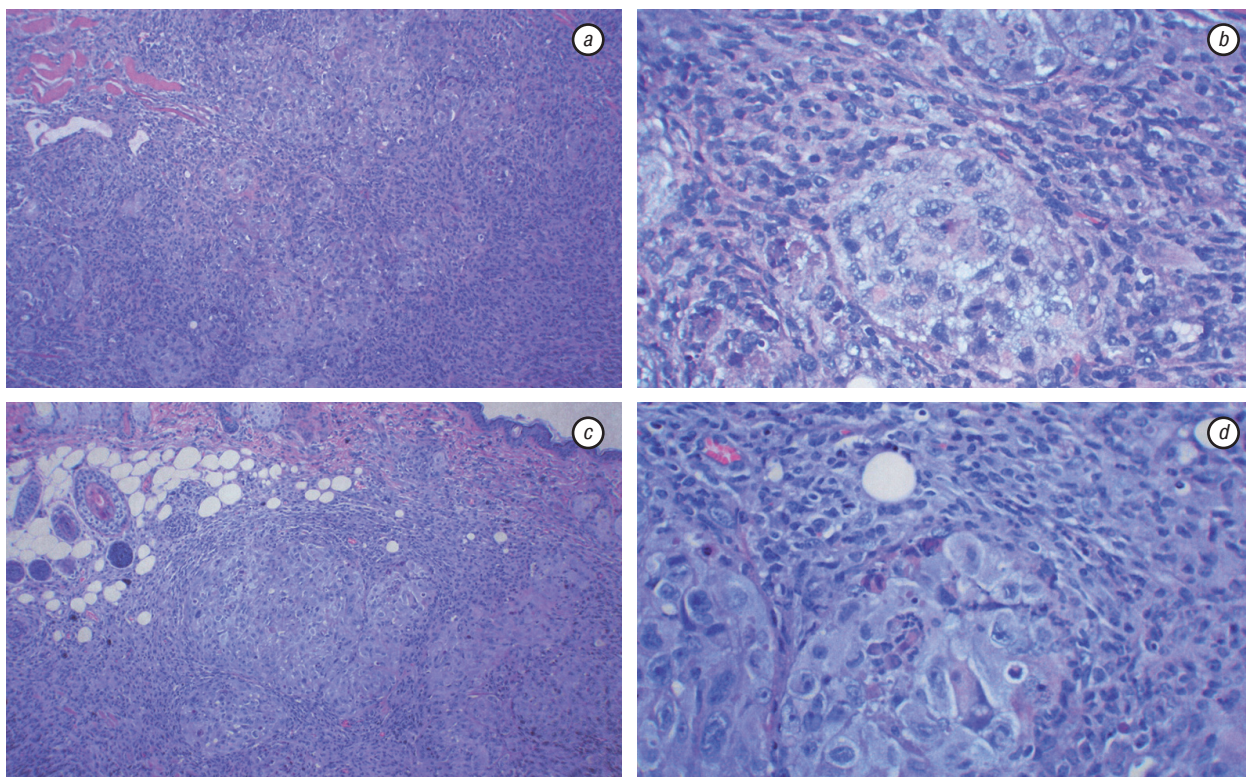


Fig. 3. Histopathology of tumors: a, control (100X); b, control (400X); c, NM-supplemented (100X); d, NM-supplemented (400X)

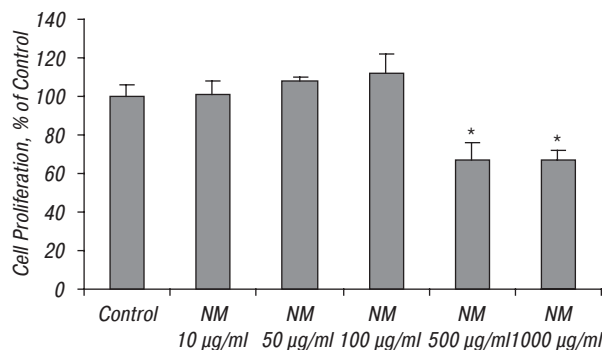


Fig. 4. Effect of NM on viability of SK-Hep-1 cells: MTT 24 h assay performed in triplicates. *Indicates significance of at least $p = 0.002$ with respect to control.

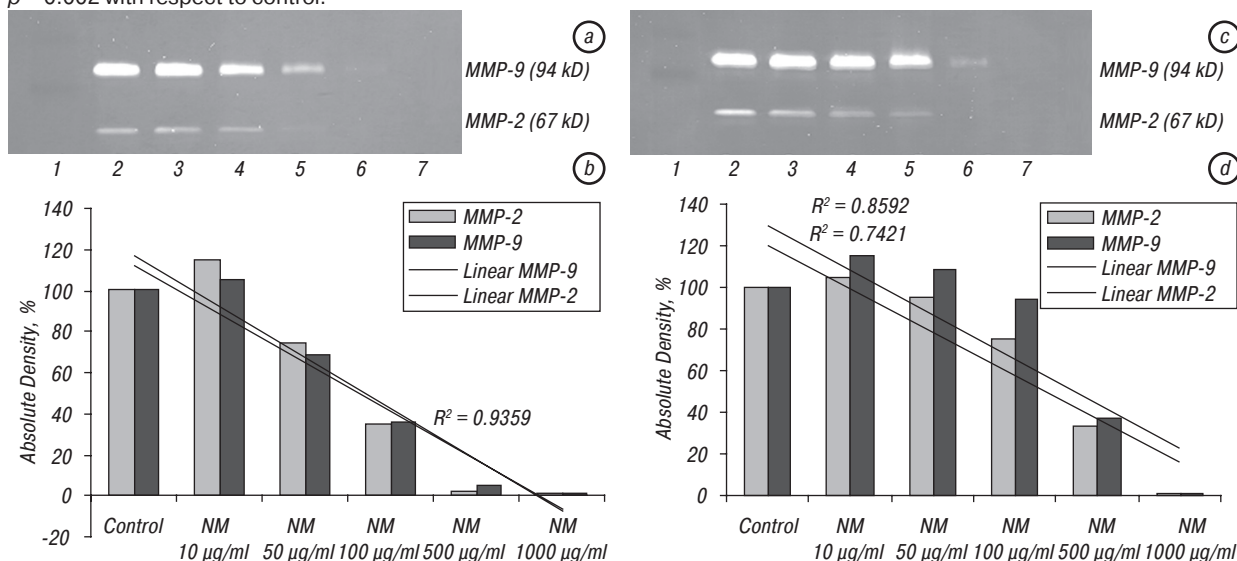


Fig. 5. Effect of NM on SK-Hep-1 cell MMP-2 and MMP-9 secretion (a) and on PMA (100 ng/ml)-treated SK-Hep-1 cell MMP-2 and MMP-9 secretion (b). 1 — markers, 2 — control, 3–7 — NM 10, 50, 100, 500, 1000 µg/ml. Densitometry analysis of untreated SK-Hep-1 cells (c) and PMA-treated SK-Hep-1 cells (d)

Gelatinase zymography. Zymography demonstrated MMP-2 and MMP-9 secretion by normal and PMA-treated SK-Hep-1 cells. NM inhibited secretion of both MMPs in a dose-dependent fashion with virtual total inhibition at 1000 µg/ml, as shown in Fig. 5, a–d.

Matrigel invasion. NM significantly inhibited SK-Hep-1 invasion through Matrigel in a dose-dependent manner, with 53% ($p = 0.0001$) inhibition at 100 µg/ml, 83% at 500 µg/ml ($p < 0.0001$) and 100% ($p < 0.0001$) at 1000 µg/ml, as shown in Fig. 6 and Fig. 7, a–f. There was significant negative correlation between NM concentration and number of SK-Hep-1 cells that invaded/migrated through Matrigel: $r = -0.8533$, $p < 0.0001$.

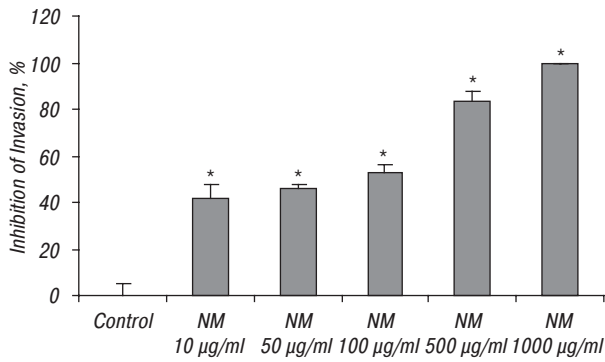


Fig. 6. Effect of NM on Matrigel invasion of SK-Hep-1 cells performed in triplicate. *Indicates significance of at least $p = 0.001$ with respect to control.

Morphology and apoptosis. H&E staining showed no morphological changes at lower concentrations but revealed significant changes at the higher concentrations of NM, as shown in Fig. 8, *a–d*. Using the live green caspase kit, dose-dependent apoptosis of SK-Hep-1 cells was evident with NM

challenge, as shown in Fig. 9, *a–d*. Approximately 16% 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. 10. At 100 µg/ml NM, 84% of cells were viable, 15% in early apoptosis and 1% in late apoptosis and at 500 µg/ml NM < 1% of cells were viable, 5% in early apoptosis, and 95% in late apoptosis. Virtually all cells exposed to 1000 µg/ml NM were in late apoptosis: < 1% viable, 2% in early apoptosis and 97% in late apoptosis.

DISCUSSION

The results of the *in vivo* study of human SK-Hep-1 xenografts in immune impaired (athymic) male nude mice demonstrated significant suppression of SK-Hep-1 tumor growth (42% reduction in tumor weight and 36% in tumor burden) with NM dietary supplementation. Results from the cellular proliferation and apoptosis studies support the *in vivo* findings, as NM showed increased toxicity in SK-Hep-1 cells with NM concentration and in-

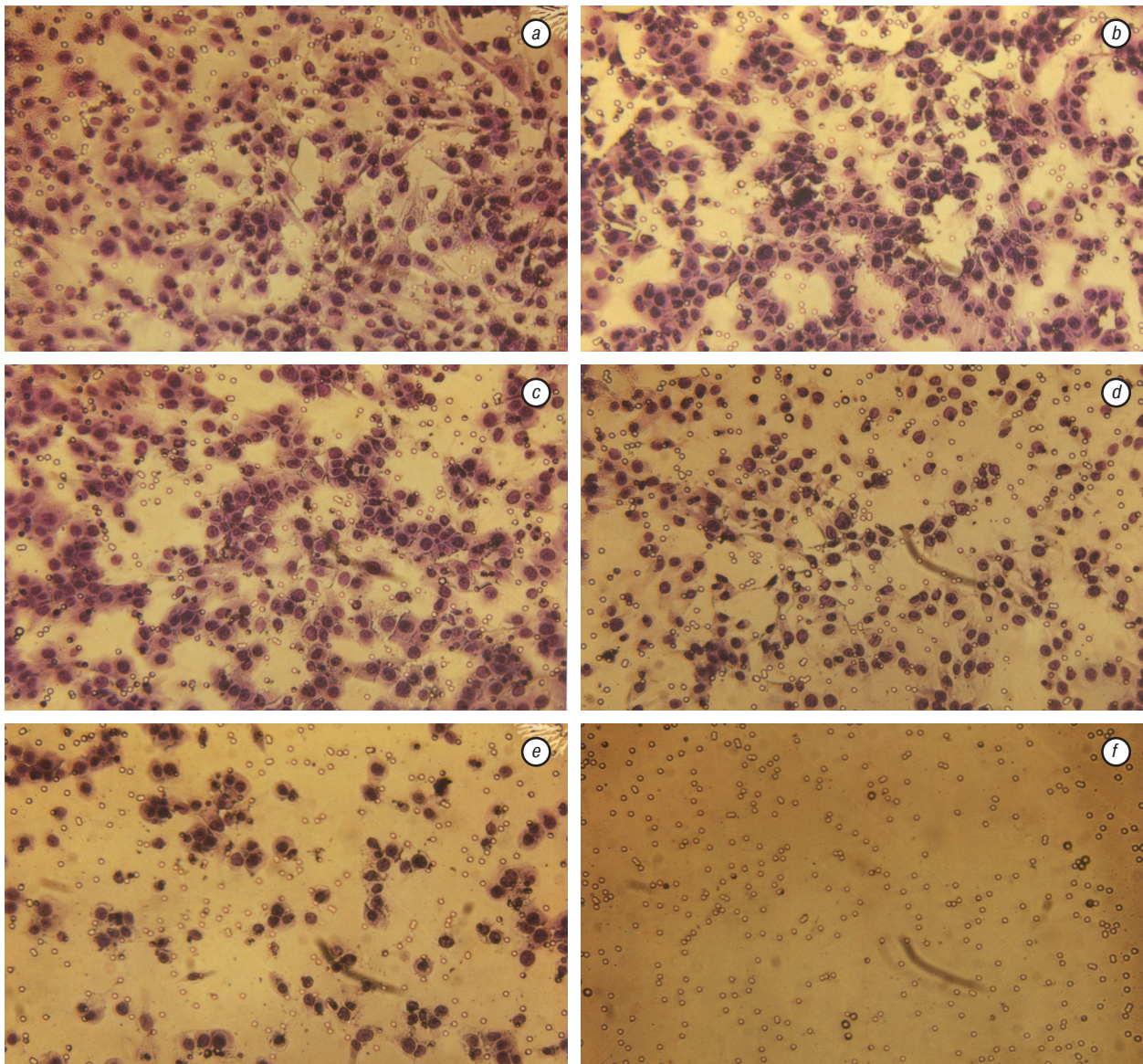


Fig. 7. Effect of NM on Matrigel invasion, photomicrographs: *a*, control; *b*, NM 10 µg/ml; *c*, NM 50 µg/ml; *d*, NM 100 µg/ml; *e*, NM 500 µg/ml; *f*, NM 1000 µg/ml

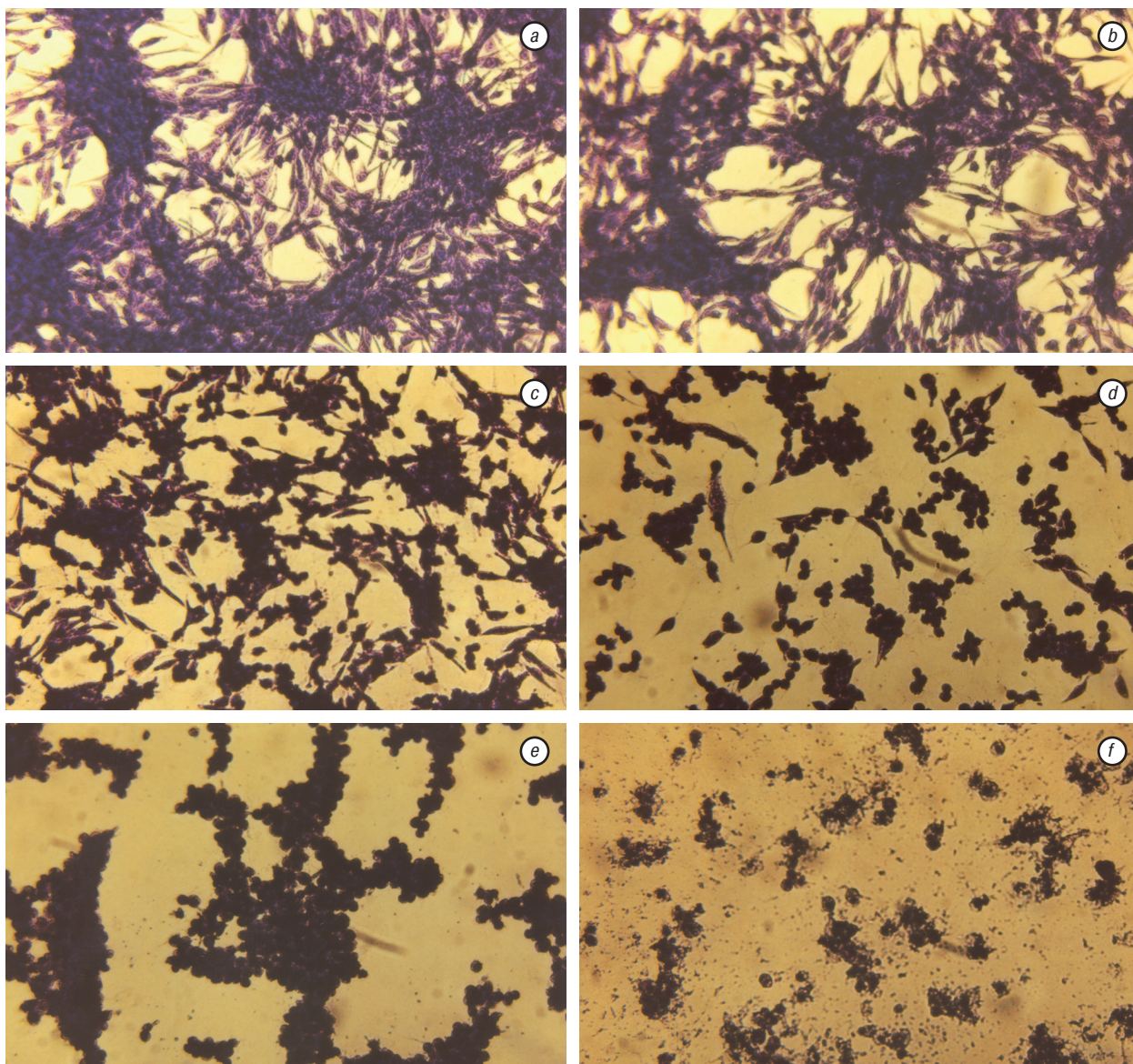


Fig. 8. Effect of NM on morphology of SK-Hep-1 cells: *a*, control; *b*, NM 10 $\mu\text{g/ml}$; *c*, NM 50 $\mu\text{g/ml}$; *d*, NM 100 $\mu\text{g/ml}$; *e*, NM 500 $\mu\text{g/ml}$; *f*, NM 1000 $\mu\text{g/ml}$. H&E staining

duced apoptosis in SK-Hep-1 cells in a dose-dependent manner, with 33% inhibition of cell growth and apoptotic induction of virtually all cells exposed to 500 $\mu\text{g/ml}$ NM.

Tumor growth depends upon the generation of new blood vessels to sustain cellular survival and proliferation. Growing tumors become hypoxic and acidotic beyond the size of 2 mm and secrete several growth factors to stimulate local blood vessels to sprout branches. Activation of endothelial cells leads to production of matrix metalloproteinases, enabling degradation of the surrounding extracellular matrix and eventual development of new blood vessels. In a previous study, NM significantly ($p < 0.05$) reduced bFGF-induced angiogenesis (utilizing a chorioallantoic membrane (CAM) assay in chick embryos, as well as decreased human osteosarcoma U2OS cell expression of VEGF, angiopoietin-2, bFGF, PDGF and TGF β -1 [11].

Invasion of host tissues is dependent on tumor cell adhesion, cell migration, and proteolytic degradation of the ECM by MMPs [25]. As mentioned previously, MMPs, especially MMP-2 and MMP-9 are prognostic for survival and metastatic potential in liver cancers.

Määttä *et al.* [6] found that MMP-2 and MMP-9 were elevated in malignant tissue and that increased latent and active MMP-2 levels predominantly were found in tumor stroma while MMP-9 was primarily found in the neoplastic epithelial cells. Furthermore, elevated MMP-2 and MMP-9 mRNA was associated with a tendency to poorer survival of patients. In examination of gelatinolytic activity in tissues of HCC patients, Sakamoto *et al.* [4] found that overexpression of MMP-9 mRNA correlated with growth of small hepatocellular carcinoma. In examining the expression of apoptotic and angiogenic factors in a group of patients with HCC, Guo *et al.* [9] found that expression of MMP-9, MMP-2 and VEGF levels positively correlated with prognosis of HCC patients, specifically with recurrence. Jiang *et al.* [5] found that high MMP-9, but not VEGF expression, correlated with recurrence or metastasis of HCC in patients post hepatectomy. In our study, MMP-2 and MMP-9 secretion and Matrigel invasion by SK-Hep-1 cells was inhibited by NM in a dose-dependent manner with virtual total block of MMP secretion and invasion at 1000 $\mu\text{g/ml}$.

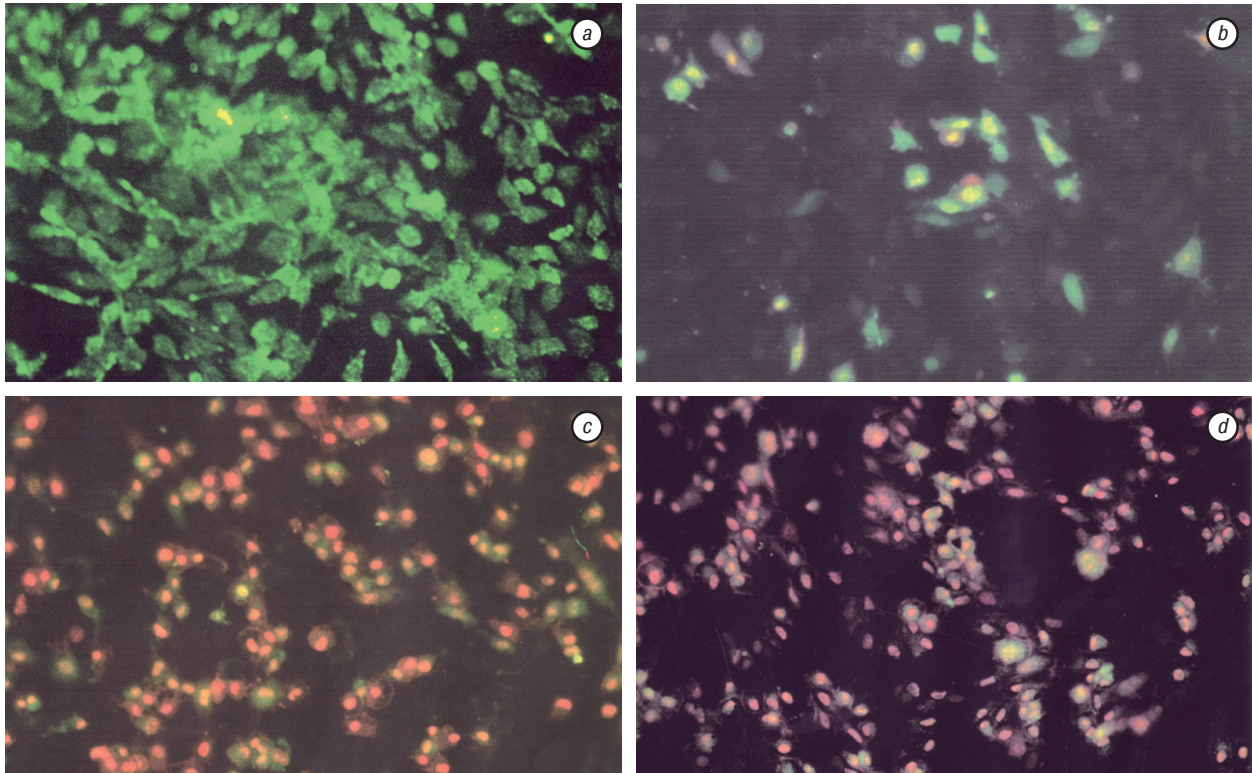


Fig. 9. Effect of NM on apoptosis of SK-Hep-1 cells: live-green caspases: a, control; b, NM 100 µg/ml; c, NM 500 µg/ml; d, NM 1000 µg/ml

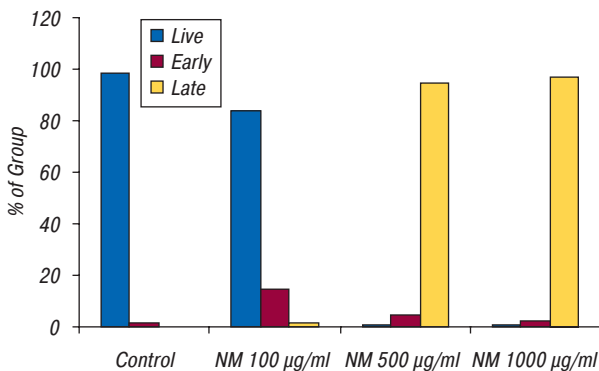


Fig. 10. Quantitative analysis of live, early and late apoptotic cells

The relative stability of extracellular matrix plays a key role in the development of cancer. We have previously shown that the ECM synthesized by normal fibroblasts treated with NM demonstrated increased stability and significantly reduced osteosarcoma cell growth rate, invasive activity (MMP-2 and MMP-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 [12].

CONCLUSIONS

The results of the present study show that supplementation with NM was effective in inhibiting growth of SK-Hep-1 xenografts in nude mice and in inhibiting growth, MMP secretion, and Matrigel invasion as well as inducing apoptosis in SK-Hep-1 cell culture. Furthermore, in contrast to the toxic side effects of current chemotherapy, the nutrient mixture has been shown to be a safe therapeutic agent. In a previous *in vivo* study addressing safety issues, we found that gavag-

ing adult female ODS rats (weighing 250–300 gm) with the nutrient mixture (at 30, 90 or 150 mg per day for seven 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.

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