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ORIGINAL PAPER

Inhibition of cell invasion and MMP production by a nutrient mixture in malignant liposarcoma cell line SW-872

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Abstract Liposarcoma, a malignancy of fat cells, is the most common soft tissue sarcoma. Though rare, poorly differentiated liposarcomas commonly metastasize to lungs and liver, leading to poor prognosis. Prevention of Extracellular matrix (ECM) degradation by inhibition of matrix metalloproteinases (MMPs) activity has been shown to be a promising therapeutic approach to inhibition of cancer progression. A nutrient mixture (NM) containing lysine, proline, ascorbic acid, and green tea extract has shown significant anticancer activity against a number of cancer cell lines. We investigated the effect of NM on liposarcoma cell line SW-872 proliferation (MTT assay), MMP secretion (gelatinase zymography), invasion through Matrigel, and apoptosis and morphology (live green caspase kit and H&E). Liposarcoma cell growth was inhibited by 36 and 61% at 500 and 1,000 μg/ml NM. Zymography demonstrated both MMP-2 and MMP-9 secretion, with PMA-enhanced MMP-9 activity. NM inhibited both MMPs with virtual total inhibition at 500 μg/ml NM. Invasion through Matrigel was inhibited at 100, 500, and 1,000 μg/ml by 44, 75, and 100%, respectively. Dose-dependent apoptosis of liposarcoma cells was evident with NM challenge, with virtually all cells exposed to 1,000 μg/ml NM in late apoptosis. H&E staining did not demonstrate any changes in morphology at lower concentrations. However, some apoptotic changes were evident at higher concentrations. In conclusion, NM significantly inhibited liposarcoma cell growth, MMP activity, and invasion and induced apoptosis in vitro—important parameters for cancer development, suggesting NM as a potential treatment strategy for liposarcoma.

Keywords Liposarcoma · MMPs · Matrigel invasion · Nutrients · Green tea extract · Ascorbic acid · Lysine

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Introduction

Liposarcoma, a malignancy of fat cells, is the most common soft tissue sarcoma in adults, slightly more frequent in males than in females, with incidence of approximately 1,000 patients in the United States per year; liposarcoma is less frequent in children with fewer than 60 cases annually. The mean patient age at onset is 50. Liposarcomas occur most commonly in the extremities and the retroperitoneal area, and less frequently in the head and neck, arising from deep-seated, well vascularized structures than from submucosal or subcutaneous fat [1].

Patients are usually asymptomatic until the enlarging tumor impinges on adjacent structures, causing tenderness, pain, or functional disturbances. Most liposarcomas have well-defined and mostly lobulated margins, while pleomorphic types have a markedly heterogeneous internal structure. The malignant grade increases with the degree of tumor heterogeneity. Well-differentiated liposarcomas and myxoid types have favorable prognosis with 100 and 88% 5-year survival rates respectively; however, these tumors tend to recur if incompletely excised, and evolve to a higher grade or dedifferentiate, leading to possible metastasis. Poorly differentiated liposarcomas have a poor prognosis with 5-year survival rate of 50% due to local recurrence and tendency to metastasize (usually to lungs and liver) quickly and widely [2].

Extracellular matrix (ECM) matrix metalloproteinases (MMPs) produced by tumor and stromal cells play a key role in tumor invasion and metastasis. Prevention of ECM degradation by inhibition of MMP activity has been shown to be a promising therapeutic approach to inhibition of cancer development. Increased MMP-2 and MMP-9 expression were significantly related to increased metastasis and poor survival in 73 liposarcoma patients studied by Benassi et al [3].

In 1992 Rath and Pauling [4] postulated that nutrients such as lysine and ascorbic acid (vitamin C) have the potential to modulate tumor growth and expansion by acting as natural inhibitors of ECM proteolysis and by stabilizing connective tissue. These nutrients can exercise their anti-tumor potential through several mechanisms, among them the inhibiting of MMPs (preserving matrix integrity) and strengthening of connective tissue surrounding cancer cells (tumor encapsulating effect). Our previous studies have confirmed this approach and demonstrated that the combination of lysine and ascorbic acid with proline, arginine, and EGCG-enriched green tea extract is effective against several cancer cell lines in blocking cancer growth, tissue invasion, angiogenesis, and MMP expression both in vitro [5–8] and in vivo [9].

In the current study, we investigated the anti-tumor potential of NM in vitro on malignant liposarcoma cell line SW-872.

Materials and methods

Cell culture

Human malignant liposarcoma cells SW-872 from ATCC (American Type Culture Collection, Rockville, MD), were grown in MEM 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% CO_2. At near confluence, the cells were treated with the nutrient mixture (NM), dissolved in media and tested at 0, 10, 50, 100, 500, and 1,000 μg/ml in triplicate at each dose. The plates were then returned to the incubator.

MTT assay

Cell proliferation 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. 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 OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

Gelatinase zymography

MMP activity in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 μl) mixed with sample buffer was loaded and SDS-PAGE was performed with tris glycine SDS buffer as described 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 $CaCl_2$ 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. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins. Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 1,200 dpi. The intensity of the bands was evaluated using a pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, by Silk Scientific Corporation (P.O. Box 533, Orem, UT 84059, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi), and expressed as a percentage of control. The R^2 value (0–1) on graphs represents how well the line of best fit falls on the dependent data, with $R^2 = 1.0$ being the best possible fit.

Matrigel invasion studies

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, human malignant liposarcoma 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_2 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.

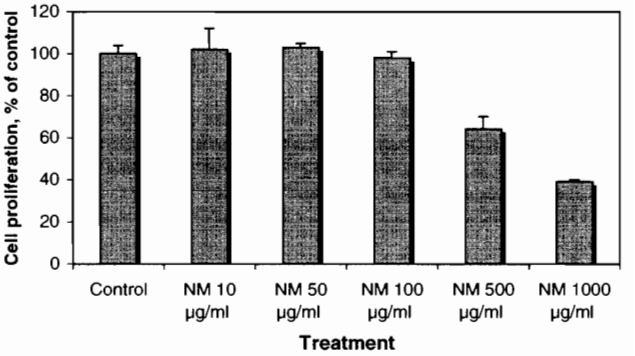

Fig. 1 Effect of NM on liposarcoma SW-872 cell proliferation: MTT assay 24 h

Apoptosis and morphology

At near confluence, liposarcoma cells were challenged with the NM dissolved in media at 0, 100, 250, 500, and 1,000 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. Morphology of cells cultured for 24 h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy.

Composition of nutrient mixture (NM)

Stock solution of the NM prepared for testing was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitoyl ascorbate) 700 mg; L-lysine 1,000 mg; L-proline 750 mg; L-arginine 500 mg; *N*-acetyl cysteine 200 mg; standardized green tea extract 1,000 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%, EGCG 35%, and caffeine 1.0%); selenium 30 µg; copper 2 mg; manganese 1 mg.

The composition of this NM was based on targeting different biological pathways involved in cancer progression and metastasis. For example, ECM integrity is dependent upon adequate collagen formation and its controlled enzymatic degradation. Therefore, the amino acids lysine and proline were included as the main components of collagen fibers and ascorbic acid, which is essential for their hydroxylation. Manganese and copper are essential cofactors in collagen and ECM formation. In addition, ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide [10]. Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [11]. *N*-acetyl cysteine has been observed to inhibit MMP-9 activity [12] and invasive activities of tumor cells [13]. Selenium has been shown to

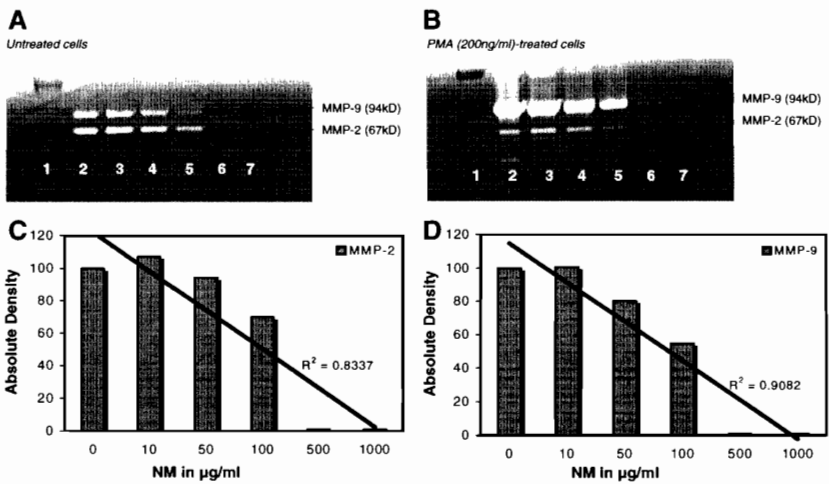

Fig. 2 Effect of NM on MMP secretion of liposarcoma SW-872 cells: (**A**) Untreated cells; (**B**) Cells treated with PMA 200 ng/ml (Legend: 1-Markers, 2-Control, 3–7 NM 10, 50, 100, 500, 1,000 µg/ml); (**C**) Densitometry analysis: effect of NM on relative activity of MMP-2 in human liposarcoma cells SW-872; (**D**) Densitometry analysis: effect of NM on relative activity of MMP-9 in human liposarcoma cells SW-872

interfere with MMP expression and tumor invasion [14], as well as migration of endothelial cells through ECM [13]. 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 [15].

Based on the evidence available in literature and our own research, we postulated that a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetyl cysteine, selenium, copper, and manganese would work synergistically. 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 [6]. Thus, by including nutrients like *N*-acetyl cysteine, arginine, selenium, manganese, and copper in

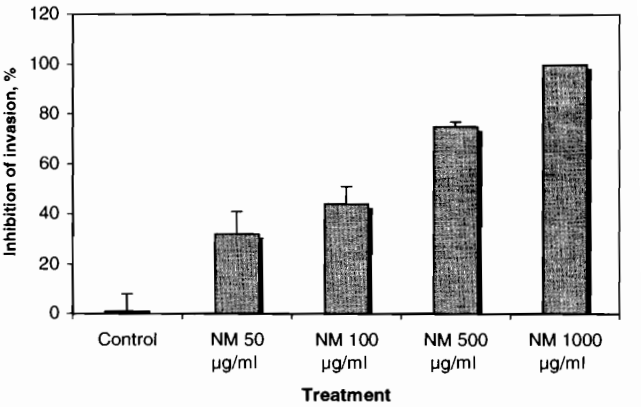

Fig. 3 Effect of NM on Matrigel invasion of liposarcoma SW-872 cells

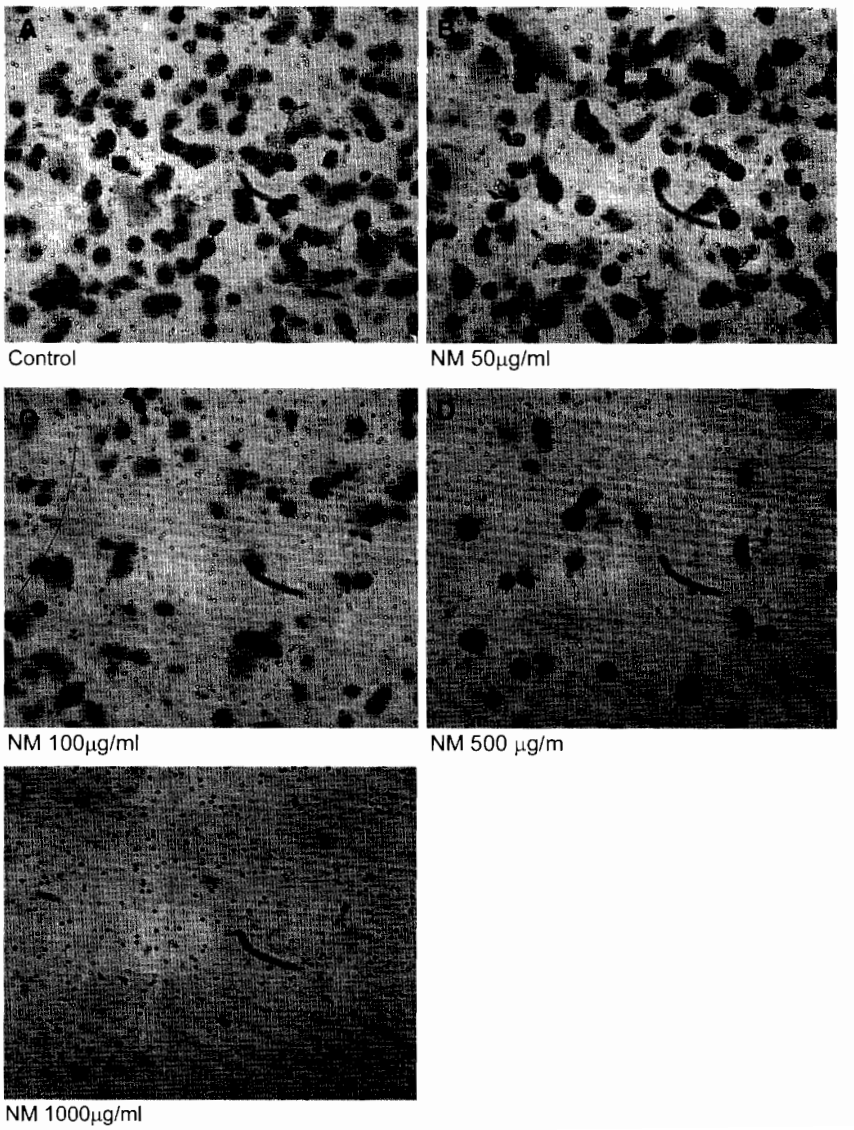

Fig. 4 Liposarcoma SW-872 invasion photomicrographs: (**A**) Control; (**B**) NM 50 μg/ml; (**C**) NM 100 μg/ml; (**D**) NM 500 μg/ml; (**E**) NM 1,000 μg/ml

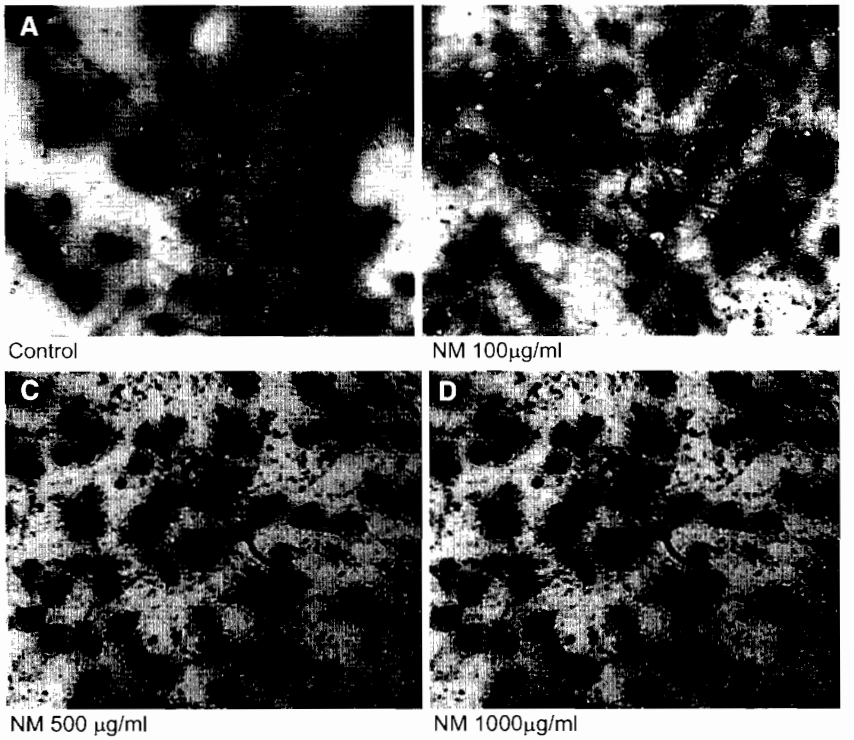

Fig. 5 Liposarcoma SW-872 cell morphology photomicrographs (H&E staining): (**A**) Control; (**B**) 100 μg/ml; (**C**) NM 500 μg/ml; (**D**) NM 1000 μg/ml

addition to ascorbic acid, proline, lysine, and EGCG we could obtain significant reduction in cell invasion at a much lower concentration of individual components.

Statistical analysis

The results were expressed as means ± SD for the groups. Data was analyzed by independent sample "*t*" test.

Results

Cell proliferation study

The NM inhibited liposarcoma cell growth by 36% at 500 μg/ml ($P = 0.005$) and 61% at 1,000 μg/ml NM ($P = 0.009$), as shown in Fig. 1.

Gelatinase zymography study

Zymography demonstrated that liposarcoma SW-872 cell line secretes both MMP-2 and MMP-9 (Fig. 2A) with increased MMP-9 secretion with PMA (200 ng/ml) treatment (Fig. 2B). NM inhibited the secretion of both MMPs in a dose-dependent fashion with virtual total inhibition at 500 μg/ml NM. Densitometry analysis on relative activity of MMP-2 showed 30% inhibition at 100 μg/ml and 99% at 500 μg/ml and 1,000 μg/ml, with $R^2 = 0.8337$ (Fig. 2C). Densitometry analysis of relative activity of MMP-9 showed 20% at 50 μg/ml, 45% at 100 μg/ml and 99% at 500 μg/ml and 1,000 μg/ml, with $R^2 = 0.9082$, as shown in Fig. 2C and D.

Invasion study

The NM significantly reduced the invasion of malignant liposarcoma cells through Matrigel in a dose-dependent fashion, with 44% inhibition at 100 μg/ml and 75% at 500 μg/ml NM and 100% at 1,000 μg/ml ($P < 0.0001$), as shown in Figs. 3 and 4.

Morphology study (Hematoxylin and Eosin staining) and apoptosis (live green caspases detection kit)

H&E staining showed no morphological changes at 100 μg/ml NM but obvious apoptotic cells were evident at 500 and 1,000 μg/ml NM (Fig. 5A–D). The apoptotic cells showed shrinkage, with condensed and darkly stained nuclei and strong acidophilic cytoplasm. Using the live green caspase kit, dose-dependent apoptosis of liposarcoma cells was evident with NM challenge, as shown in Fig. 6A–E. Slight apoptosis of liposarcoma cells was observed in cells exposed to 100 μg/ml and 250 μg/ml NM, moderate at 500 μg/ml and profound at 1,000 μg/ml. Quantitative analysis of live, early and late apoptotic cells is shown in Fig. 7. Moderate apoptosis with 63% live cells and 37% in

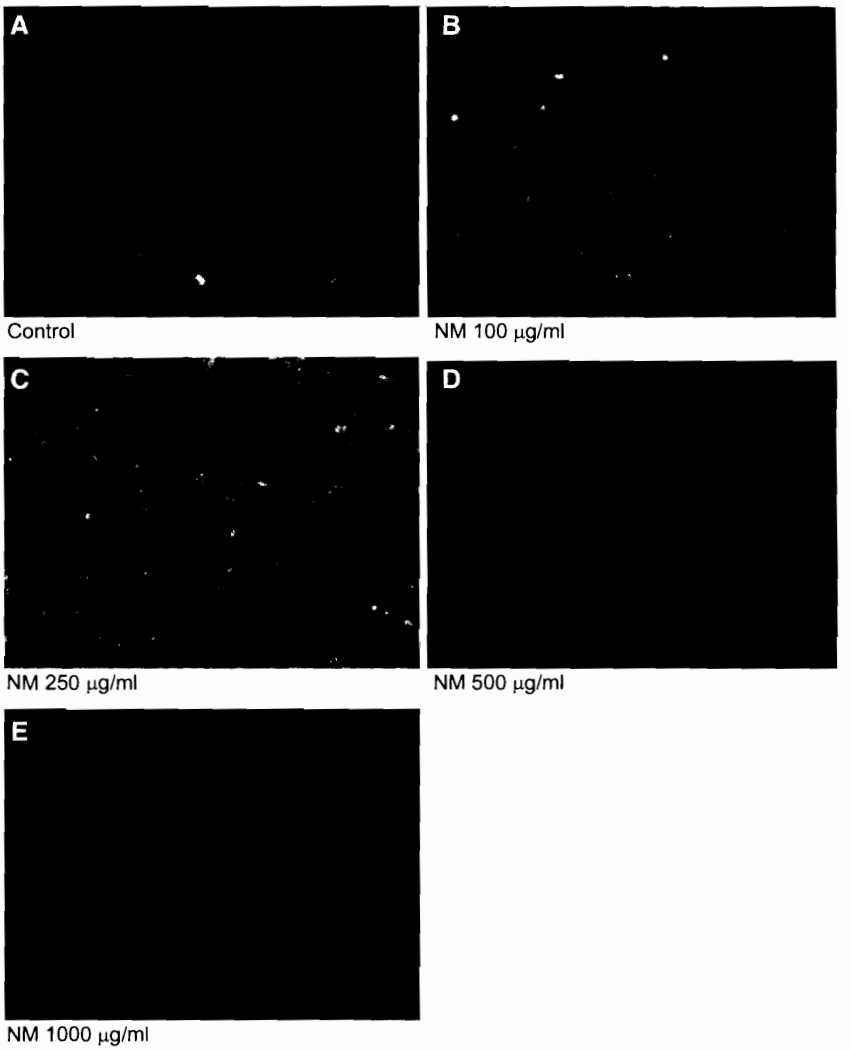

Fig. 6 Induction of apoptosis in liposarcoma SW-872 cells by NM photomicrographs (live green caspase detection kit): (**A**) Control; (**B**) NM 100 μg/ml; (**C**) NM 250 μg/ml; (**D**) NM 500 μg/ml; (**E**) NM 1,000 μg/ml

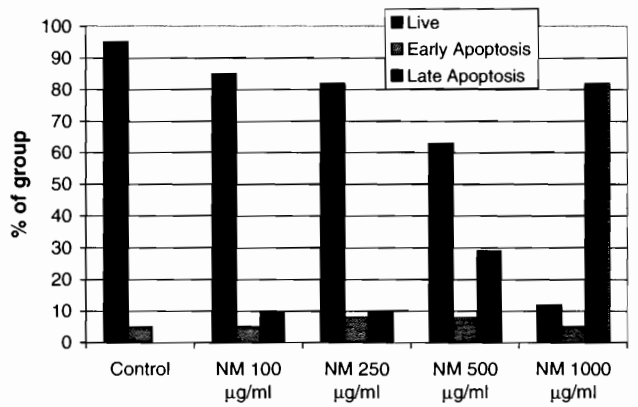

Fig. 7 Percent of liposarcoma SW-872 cells in stages of apoptosis with NM treatment

early or late apoptosis was seen in cells exposed to 500 μg/ml NM. Virtually all cells exposed to 1,000 μg/ml NM were in late apoptosis.

Discussion

The NM demonstrated significant inhibition of human malignant liposarcoma SW-872 cell invasive parameters in vitro. Matrigel invasion and MMP-2 and MMP-9 secretion of liposarcoma cells decreased in a dose-dependent fashion with complete inhibition of MMP-2 and -9 at 500 μg/ml, and of Matrigel invasion at 1,000 μg/ml. In addition NM demonstrated dose-dependent antiproliferative and pro-apoptotic effects on liposarcoma cells with 61% inhibition of cell proliferation at 1,000 μg/ml and profound induction of apoptosis and morphological changes at that concentration.

As mentioned previously, increased MMP-2 and MMP-9 levels in liposarcoma patient biopsies have been shown to be associated with increased metastasis and poor survival [2]. In addition, MMP-2 and -9 expression by liposarcoma SW-872 appears to be induced by growth factors. In studying the effect of interleukin-1α on MMP activity of

human liposarcoma cell line SW-872 in vitro. Pazzaglia et al [16] observed that exposure of S100 A-4-positive SW872 cells to interleukin-1α, increased MMP-9 and MMP-2 activity. In a previous study, we found that NM inhibited cytokine secretion [17] as well as cytokine-induced MMP secretion by tumor cells [18]. Furthermore, cultured human liposarcoma cells develop into a variety of subpopulations with different angiogenic activities. In studying the effect of human liposarcoma cells SW-872 xenografts in severe immune deficient mice, Achilles et al [19] found that this single type of human tumor contains subpopulations of tumor cells with different angiogenic activities and thus tumor volume. In previous studies we found that NM suppressed angiogenesis and angiogenic-promoting growth factors [8].

Control of proteolytic activity of ECM provides an opportunity of addressing common mechanisms of metastasis, angiogenesis, and tumor growth. Rath and Pauling [3] suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogues. Plasmin is formed upon cleavage of the zymogen plasminogen by plasminogen activators, urokinase-type plasminogen activator (uPA), and tissue-type plasminogen activator (tPA). Lysine prevents the activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, and consequently prevents the plasmin-induced MMP activation cascade [3]. A recent study confirmed the effectiveness of this approach by showing a 7-fold reduction in metastasis of transgenic mammary cancer in plasmin deficient mice [20].

The inhibitory effects of lysine, proline, ascorbic acid, and green tea extract on cancer development have been reported in clinical as well as experimental studies. Ascorbic acid has been reported to have cytotoxic and antimetastatic effects on various malignant cell lines [21–25]; in addition, low levels of ascorbic acid have been reported in cancer patients [26]. Green tea extract is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines [27–29].

Conclusions

While animal and clinical studies are necessary to better determine the efficacy of this nutrient formulation in both liposarcoma prevention and treatment, the results of this study suggest that NM is a promising candidate for therapeutic use by inhibiting liposarcoma cell growth, MMP activity and invasion and inducing apoptosis.

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