

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

M. W. Roomi · V. Ivanov · T. Kalinovsky ·
Aleksandra Niedzwiecki · M. Rath

Received: 26 March 2007 / Accepted: 11 April 2007 / Published online: 25 May 2007
© Humana Press Inc. 2007

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

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].

M. W. Roomi · V. Ivanov · T. Kalinovsky ·
A. Niedzwiecki (✉) · M. Rath
Cancer Division, Dr. Rath Research Institute, 1260 Memorex
Drive, Santa Clara, CA 95050, USA
e-mail: a.niedz@drath.com

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% CO₂. 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 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

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 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. 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% 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.

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

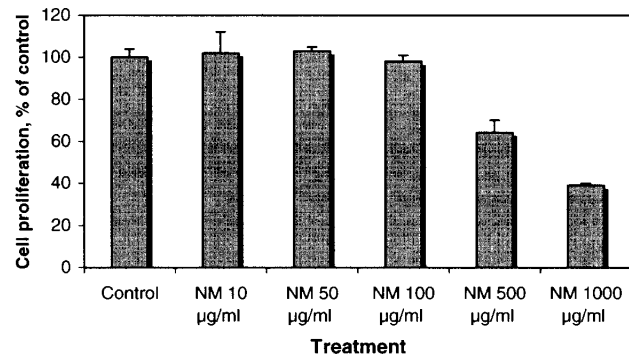


Fig. 1 Effect of NM on liposarcoma SW-872 cell proliferation: MTT assay 24 h

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

