The Anti-Cancer Effect of a Novel Nutrient Mixture by Inhibiting MMPs Expression, Invasion and Inducing Apoptosis in Chondrosarcoma Cell Line SW-1353

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Abstract

Chondrosarcoma, a malignant tumor of cartilaginous origin is the most frequent adult primary bone cancers. Surgery is the main treatment option because chondrosarcoma typically does not respond to radiation and chemotherapy. Cancer mortality usually results from the local and distant metastasis. A nutrient mixture containing ascorbic acid, lysine, proline, green tea extract, was tested on chondrosarcoma cells SW-1353 for viability, matrix metalloproteinase expression, Matrigel invasion, morphology and apoptosis. The SW-1353 cells were grown in appropriate media and treated with the nutrient mixture at 10, 50, 100, 500 and 1000 µg/ml concentrations. Cells were also treated with PMA (100 ng/ml) for MMP-9 stimulation. Cell proliferation was carried out by MTT assay, MMPs by zymography, invasion through Matrigel. Morphology and apoptosis were also conducted. The nutrient mixture did not exhibit toxicity at 100 µg/ml, but showed 40% inhibition at 1000 µg/ml. Zymography demonstrated two bands, for MMP-2 and MMP-9. PMA treatment further enhanced MMP-9 expression. The nutrient mixture inhibited expression of both MMPs in a dose dependent manner. Matrigel invasion was reduced by 40%, 70%, 88% and 100% at 50, 100, 500 and 1000 µg/ml respectively. The nutrient mixture induced slight apoptosis at 250 µg/ml, moderate at 500 µg/ml and significant at 1000 µg/ml concentrations. Morphology showed slight changes at the highest concentrations. The nutrient mixture significantly inhibited all the important hallmarks for cancer progression, suggesting that it has a possibility for exploration as a significant therapeutic entity in chondrosarcoma.

Keywords: chondrosarcoma, nutrient mixture, MMP, Matrigel invasion, apoptosis

1. Introduction

Chondrosarcoma, a malignant tumor of cartilage cells mainly affecting adults between 30-60 years. According to the American Cancer Society 2012 estimates, chondrosarcoma has become the most common primary bone cancer accounting for more than 40% of the adult primary bone tumor cases. It grows in the chondrocytes (cartilage cells), and mainly affects the legs, upper arms, scapula, ribs and pelvic bones. Although the exact cause of chondrosarcoma is not known, it is postulated that individuals with certain genetic or chromosomal abnormalities are at increased risk. Grade and stage are the most important and independent prognostic factors for survival in chondrosarcoma. Cancer mortality usually occurs from tumor invasion resulting in local or distant metastasis to vital organs. High-grade chondrosarcoma has much poorer prognosis. 10-year survival of high-grade chondrosarcoma was calculated to be 38% (Fiorenza et al., 2002). Recurrence is based on irregular tumor margins and lesions greater than 10 cm. Surgery is generally the main treatment option for chondrosarcoma. Chemotherapy or radiation therapies are not very effective and are rarely given in cases of extensive metastasis (Gelderblom et al., 2008). The high metastatic potential and inadequate treatment methods leading to poor outcomes, require an urgent need to develop more effective less toxic treatment alternatives.

We have developed strategies to inhibit cancer growth and expansion using naturally occurring nutrients including lysine, proline, ascorbic acid, green tea extract, and others. According to Rath and Pauling research (1992), nutrients such as lysine and ascorbic acid have been suggested to act as natural inhibitors of extra cellular matrix (ECM) degradation, and as such have potential to modulate tumor growth and expansion. These nutrients can exert their effects by strengthening the connective tissue surrounding cancer cells by increasing collagen
synthesis, as well as inhibit the expression of matrix metalloproteinase (MMP) enzymes. In our previous studies, these nutrients (the nutrient mixture, NM) have exhibited broad spectrum therapeutic and chemoprotective activities in vitro and in vivo in a number of cancer cell lines. This synergistic anticancer effects of the NM was observed by inhibition of cancer cells growth, expression of MMPs, Matrigel invasion, metastasis, and angiogenesis (Roomi et al., 2006, 2007, 2009a, 2009b). Considering the efficacy of NM on other cancer cell lines, we investigated the effects of NM on the human chondrosarcoma cell line SW-1353. We hypothesized that NM would significantly inhibit the growth, MMP expression and invasion of the SW-1353 cancer cell line.

2. Materials and Methods

2.1 Composition of Nutrient Mixture

The composition of the nutrient mixture (NM) and the proportion included the following: Vitamin C (as ascorbate salts of Mg, Ca and plamitate) 710 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 µg; Copper 2 mg; and Manganese 1 mg.

2.2 Cancer Cells and Culture

Chondrosarcoma cells line, SW-1353 was from American Type Culture Collection (ATCC, Rockville, MD), and grown in modified Dulbecco’s Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Long Island, NY) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated in 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 NM, dissolved in the media and tested in triplicate: 0, 10, 50, 100, 500 and 1000 µg/ml. Cells were also treated with Phorbol Myristate Acetate (PMA) 100 ng/ml to enhance MMP-9 expression. The plates were then returned to the incubator.

2.3 MTT Assay

Cell viability was carried out by MTT assay. The 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 tetrazilium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This method is a good index of mitochondrial activity and thus of cell viability. After a 24 hour incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma#M-2128) 0.5mg/ml in media was added. After addition the plates were returned to the 37°C incubator for 2 hours. After which, the supernatant was removed, 1 ml of DMSO was added, and absorbance was read 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 OD of the control (treatment without supplement) was considered 100%.

2.4 Gelatinase Zymography Enzyme Activity Assay

Gelatinase zymography was carried out in 10% Novex Pre-Case 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 Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as suggested by the manufacturer (Novex). Samples were not heated before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes to remove SDS. The gels were then incubated at 37°C overnight in CaCl2 at pH 8.0 and stained with 0.5% Coomassie Blue R250 dissolved in 50% methanol and 10% glacial acetic acid 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 also ran and approximate molecular weights were calculated. 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, (Silk Scientific Corporation) at a resolution of 1 Scanner Unit (1/100 dpi), and expressed as a percentage of control.

2.5 Matrigel Invasion Assay

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, SW-1353 cells were supplemented with the NM and seeded into the inserts in the well. Thus, both the medium on the insert and in the well had the same supplements. The plates were then returned to the incubator and equilibrated with 95% room air and 5% CO2. After incubation, the media from the wells were drawn. 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 (H & E) and counted under the microscope.
2.6 Morphology and Apoptosis
Morphology of the cells cultured in test concentrations of were evaluated after 24 hours by H&E staining and apoptosis using Live Green Caspase Detection Kit and photographed by microscope. The SW-1353 cells were challenged with NM dissolved in media at the experimental doses and incubated for 24 hours. The cell culture was washed with PBS and treated with the caspase reagent as suggested by the manufacturer (Molecular Probes Image-IT Live Green Poly Caspases Detection Kit 135104, Invitrogen). The photographs were taken with a fluorescence microscope and cells were counted. Green-colored cells represent viable cells, while yellow orange and red represents early and late apoptosis respectively.

2.7 Data Analysis
The results were expressed as mean ± standard deviation. Data was analyzed by independent t-test. Significance was determined at p<0.05.

3. Results
3.1 Cell Proliferation
Figure 1 shows the effects of NM relative to control in triplicate of the Chondrosarcoma cell line. NM was not toxic to chondrosarcoma cell line SW-1353 at 100 µg/ml, but exhibited 10% and 40% (p<0.001) toxicity at both 500 and 1000 µg/ml, respectively.

![Figure 1. Effect of the NM on chondrosarcoma cell line SW-1353 proliferation](https://example.com/figure1.png)

The cell proliferation was inhibited 10% at 500 µg/ml and 40% at 1000 µg/ml. (* Significant at p<0.001)

3.2 Gelatinase Zymography
Zymography demonstrated two bands corresponding to MMP-2 and MMP-9. PMA (Phorbol Myristate Acetate) treatment stimulated MMP-9 expression. NM inhibited the expression of both MMP-2 and MMP-9 in a dose-dependent fashion; 50% at 100 µg/ml and 100% at 500 µg/ml (Figure 2A and 2B). Densitometry analysis of MMP-2 for untreated SW-1353 cells showed inhibition of 56% at 100 µg/ml and 98% at 500 µg/ml of NM. Regression analysis showed a linear trend of R²=0.85 (Figure 2C). For SW-1353 cells treated with PMA, MMP-2 inhibition was seen as follows: 97% at 50 µg/ml, 98% at 100 µg/ml and 100% at 500 µg/ml. Regression was noted at R²=0.81 (Figure 2D). Untreated SW-1353 cells showed an inhibition of MMP-9 expression of 33% at 10 µg/ml, 98% at 50 µg/ml, 99% at 100 µg/ml and 100% at 500 µg/ml. R² for these cells was 0.88 (Figure 2C). Finally, for the SW-1353 cells treated with PMA, an equally strong inhibition of expression of MMP-9 was observed. MMP-9 expression was inhibited by 82% at 50 µg/ml of NM, 83% at 100 µg/ml and 99% at 500 µg/ml. Regression coefficient for PMA-induced MMP-9 expression of SW-1353 cells was calculated to be R²=0.86 (Figure 2D).
2A- Normal SW-1353 cells

The upper row corresponds to MMP-9 secretion and the lower line to MMP-2. Each column corresponds to increasing concentration of NM, (Lane 1: Marker, 2: Control, Lanes 3-7: NM doses 10, 50, 100, 500 and 1,000 µg/ml)

2B- SW-1353 cells treated with PMA

2C. Densitometry of MMP-2 and MMP-9 of SW-1353 cells. R² represents a linear regression analysis of the dose-dependent inhibition of MMP

2D. Densitometry analysis of MMP-2 and MMP-9 of PMA (100 ng/ml) treated SW-1353 cells

Figure 2. Effect of NM on MMP-2 and MMP-9 secretion by chondrosarcoma cell line SW 1353
3.3 Matrigel Invasion

Figure 3 (A-E) reveals a significant dose-dependent inhibition of SW-1353 cell migration and invasion through Matrigel membrane. 38% inhibition was observed at 50 µg/ml, 65% at 100 µg/ml, 85% at 500 µg/ml and 100% inhibition was noted at 1000 µg/ml of NM concentration, p<0.001 (Figure 3F).

3A: Control  
3B: NM 50µg/ml  
3C: NM 100µg/ml  
3D: NM 500µg/ml  
3E: NM 1000µg/ml

3F: Effect of NM Matrigel invasion on SW-1353 cells (* Significance p<0.001 with respect to control)

3.4 Morphology and Apoptosis

H&E staining of SW-1353 cells exposed to NM showed no changes at the 50 µg/ml and 100 µg/ml doses but showed slight changes at the 500 µg/ml dose (Figure 4 A-E). These changes include, shrinking of the cytoplasm,
darkly stained nuclei and intensely acidophilic cytoplasm suggest cells undergoing apoptosis. Using the live green caspase kit, a dose-dependent apoptosis of the SW-1353 cells was evident with the NM. Figure 5 (A-E) highlights that as the concentration of NM was increased the apoptotic events also increased. A quantitative analysis of this is represented in Figure 5F, which shows concentration of live cells gradually decreased as the NM dose increased. At 250 µg/ml of NM, 81% cells were live, however at 500 µg/ml only 37% cells were live and 62% were either in early or late apoptosis. At 1000 µg/ml, 10% cells were live, while 90% cells were in early and late apoptosis stage.

Figure 4. SW 1353 cell morphology showed slight changes at the higher concentrations

Figure 5. SW 1353 cell apoptosis analysis at different concentrations of NM.
4. Discussion

The results of our experiments on the chondrosarcoma cell line SW-1353 suggest that NM is effective in inhibiting cell proliferation above a concentration of 500 µg/ml. Additionally, a dose-dependent decrease in MMP expression and Matrigel migration was observed. Furthermore, NM also induced apoptosis. These are the most important steps in cancer invasion and metastasis.

The NM formulation was based upon several important nutrients that exhibited anti-cancer properties, in particular those that reinforce the ECM, inhibit MMP activity and are cytotoxic to cancer cells. For example, ascorbic acid and the amino acids lysine and proline are critical to proper ECM formation and structural integrity by ensuring synthesis and hydroxylation of collagen fibers (Roomi et al., 2005). Lysine possesses an additional role in maintaining proper collagen formation by inhibiting proteolysis (Rath & Pauling, 1992). Green tea extract has shown promise in controlling cancer growth, metastasis and angiogenesis (Taniguchi et al., 1992; Valcic et al., 1996; Mukhtar & Ahmed, 2000). N-acetylcycteine is known to inhibit MMP-9 and invasive activities of tumor cells (Morini et al., 1999; Kawakami, Kageyama, Fujii, Kihara, & Oshima, 2001). In addition to regulating ECM properties, certain nutrients can induce cell death. Ascorbic acid, for example, is known to inhibit cell division and growth via hydrogen peroxide production (Maramag, Menon, Balaji, Reddy, & Laxmanan, 1997). Arginine, a known precursor of nitrogen oxide, also plays an important role in cell apoptosis induction (Cooke & Dzau, 1997). Finally, selenium has been shown to inhibit the secretion of MMP secretion and tumor invasion (Yoon, Kim, & Chung, 2001).

These nutrients can exert their effects by strengthening the connective tissue surrounding cancer cells by influencing collagen synthesis, as well as inhibit the expression of MMP enzymes. Free radical injury plays a key role in cancer initiation and progression. During the multistep process, the degradation of ECM by MMPs is a critical step in tumor growth, invasion, and metastasis. It is important to restrict this step to halt tumor progression. Matrix metalloproteinases are a family of proteolytic enzymes able to degrade connective tissue and are associated with cancer metastasis and tumor angiogenesis. Two key enzymes, MMP-2 and MMP-9, play a key role in the degradation of collagen types II and IV, important components of the ECM. Higher expression of MMP enzymes is shown to play a role in the malignancy potential of chondrosarcomas. When high-grade chondrosarcoma samples were compared to lower-grade samples using immunohistochemistry, the ratio (grade 3 to grade 1) of gene expression of MMP-2 exceeded (Boeuf, Bovée, Lehner, Hogendoorn, & Richter, 2010). In a comparison of MMP-2 and MMP-9 expression of normal cartilage and chondrosarcoma tissue, the chondrosarcoma cells samples had a higher expression of MMPs (Moses & Shing, 1994). Elliot et al. (2002)
showed that Bcl-3, inhibitor of NF-kappaB1 can cooperate to activate MMP-1 transcription of chondrosarcoma cells SW 1353. In another study, Elliot’s group also showed that synthetic triterpenoid, CDDO, inhibited IL-1 induced MMP-1 and MMP-13 expression in SW-1353 cells (Elliott et al., 2003). While Burrag et al. evaluated the effect of synthetic ligand LG 100268 for nuclear hormone receptor and it selectively inhibit IL-1 and stimulate MMP-1 and MMP-13 (Burrag et al., 2007).

Our studies demonstrated significant inhibition of MMP-2 and MMP-9 expression, the important mediators of angiogenesis and metastasis. These data suggest NM may have a role in the therapeutic approaches of chondrosarcoma, specifically by targeting MMP expression and thereby inhibiting migration of chondrosarcoma within the ECM as well as stabilizing the ECM surrounding an encapsulated tumor, thereby reducing chances of metastasis. In addition, NM maximally inhibits the proliferation of cancer cells at high doses, inhibits Matrigel invasion and induces apoptotic changes at the cellular level. The effect appears to be cancer-specific since our previous studies demonstrated no NM toxicity on a variety of normal cells, such as fibroblasts, smooth muscle cells and endothelial cells (Ivanov, Ivanova, & Niedzwiecki, 1997; Ivanov et al., 2007). Furthermore, the NM has also been shown to be safe in vivo. In a previous in vivo study, we found that gavaging adult female ODS rats with the NM (at 3, 90 and 150 mg per day for seven days), had no adverse effects on vital organs such as the heart, liver, and kidneys. In the same study, the NM did not affect the associated serum enzymes indicating that this mixture is safe to use even at higher doses, by far exceeding the normal equivalent doses of this combination (Roomi et al., 2003). Overall, the NM may offer a therapeutic benefit and play a role in support of chondrosarcoma.

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References


