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INHIBITION OF MALIGNANT MESOTHELIOMA CELL MATRIX METALLOPROTEINASE PRODUCTION AND INVASION BY A NOVEL NUTRIENT MIXTURE

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☐ *Malignant mesothelioma (MM), an asbestos-associated cancer with no known cure, is a highly aggressive tumor causing profound morbidity and nearly universal mortality. 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 MMP inhibition has been shown to be a promising therapeutic approach to inhibition of cancer development. Based on reported anticancer properties, the authors investigated the effect of a mixture (NM) containing lysine, proline, ascorbic acid, and green tea extract on MM cell line MSTO-211 H proliferation (by [MTT] [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay), MMP secretion (by gelatinase zymography), invasion (through Matrigel), and morphology (by hematoxylin and eosin [H&E] staining). MMP-2 and phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 secretion were inhibited by NM in a dose-dependent fashion, with virtual total inhibition at 500 μg/ml NM. Invasion through Matrigel was inhibited at 50, 100, and 500 μg/ml by 27%, 36%, and 100%, respectively. NM was not toxic to the MM cell line, and H&E staining did not indicate any changes at and below 100 μg/ml concentration. In conclusion, NM significantly inhibited MM cell MMP secretion and invasion—both important parameters for cancer prevention, suggesting NM is an effective treatment strategy for MM.*

Keywords ascorbic acid, green tea extract, lysine, MMPs, malignant mesothelioma, matrigel invasion, nutrients

Malignant mesothelioma is a highly aggressive tumor that arises from mesothelial-lined surfaces, most frequently in the pleural cavities. Median survival for patients with malignant pleural disease has been reported to be 4 to 18 months [1]. Necroscopic examination has shown metastatic spread in 50% of patients [2]. Exposure to asbestos is the primary cause for this disease. Epidemiological evidence suggests that due to the heavy

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use of asbestos until 1980, the number of men in Western Europe dying each year will increase from 5000 in 1998 to 9000 in 2018 [3]. Between 2000 and 3000 cases are diagnosed in the United States each year [4].

The natural history of malignant mesothelioma involves aggressive local growth, invasion of vital mediastinal structures, and death within 4 to 12 months without treatment. Single- and multiple-modality therapies (surgical resection, chemotherapy, and radiotherapy) have failed to substantially alter this natural history [1]. There is obviously a need for safe and effective alternative approaches that can be used to control the aggressive spread of mesothelioma.

Proteolytic degradation of extracellular matrix and basement membranes are key components of tumor cell invasion and metastasis. All types of cancer cells form tumors and spread in the body by degrading the extracellular matrix (ECM) by means of various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. Previous studies have shown that malignant mesothelioma cells express MMP-1, -2, -3, -7, and -9 [5].

In 1992 Rath and Pauling postulated that nutrients such as lysine and ascorbic acid (vitamin C) could act as natural inhibitors of ECM proteolysis and, therefore, by stabilizing connective tissue, have the potential to modulate tumor growth and expansion [6]. These nutrients can exercise their antitumor potential through several mechanisms, among them the inhibiting of MMPs and strengthening of connective tissue surrounding cancer cells (tumor encapsulating effect).

In this study, we investigated the antitumor potential of the nutrient mixture (NM) in vitro on the malignant mesothelioma MSTO-211 H cell line.

MATERIALS AND METHODS

Cell Culture

Human malignant mesothelioma MSTO-211H, obtained from ATCC (American Type Culture Collection, Rockville, MD), was grown in RPMI 1640 medium, 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 medium 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 medium, and tested at 0, 10, 50, 100, 500, and 1000 µg/ml in triplicate at each dose. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) 200 ng/mL to induce MMP-9 secretion. The plates were then returned to the incubator.

MTT Assay

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (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 catalog no. M-2128), 0.5 mg/mL in medium was added to each well. The plates were covered and returned to the 37°C incubator for 2 hours, 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 Shimadzu Bio Spec 1601 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 medium was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast sodium dodecyl sulfate (SDS)-polyacrylamide gel (Invitrogen) in the presence of 0.1% gelatin under nonreducing conditions. Culture medium (20 µL) mixed with sample buffer was loaded and SDS–polyacrylamide gel electrophoresis (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 minutes 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, stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes, and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Matrigel Invasion Studies

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, human malignant mesothlioma 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 hours. 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 (H&E) and visually counted under the microscope.

Composition of Nutrient Mixture (NM)

Stock solution of the nutrient mixture (total weight 4.2 g) prepared for testing was composed of the following: 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*-acetylcysteine 200 mg; standardized green tea extract 1000 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%, epigallocatechin gallate [EGCG] 35%, and caffeine 1.0%); selenium 30 μg; copper 2 mg; manganese 1 mg.

The nutrient mixture (NM) was formulated based on targeting different physiological processes involved in cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation and its stability; ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimal structure of collagen fibers. Manganese and copper are also essential cofactors in collagen formation. Collagen stability can be controlled by lysine [6] and also by *N*-acetylcysteine through its inhibitory effect on MMP-9 activity [7] and invasive activities of tumor cells [8]. Also selenium has been shown to interfere with MMP expression and tumor invasion [9], as well as migration of endothelial cells through ECM [8]. Ascorbic acid has also 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]. Because 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 [12].

Based on the evidence available in literature and our own research, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetylcysteine, selenium, copper, and manganese would result from their synergy. 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 [13]. Thus by including *N*-acetylcysteine, arginine, selenium, manganese, and copper with ascorbic acid, proline, lysine, and EGCG,

we could obtain significant reduction in cell invasion at a much lower concentration of EGCG or other component. Also, the combined effects of these individual nutrients on decreasing proliferation of neoplastic cells were superior to the effects of their individual components or when they were randomly combined [14].

Statistical Analysis

The results were expressed as means $\pm$SD for the groups. Data was analyzed by independent-sample t test.

RESULTS

Cell Proliferation Study

NM was not toxic to the mesothelioma cell line, as shown in Figure 1. The slight decrease ($<10\%$) in cell proliferation in the presence of 100, 500, and 1000 µg/mL NM was not significant ($P = .47$).

Gelatinase Zymography

Zymography demonstrated MMP-2 and PMA (200 ng/mL) induced MMP-9 secretion by malignant mesothelioma cells. NM inhibited secretion

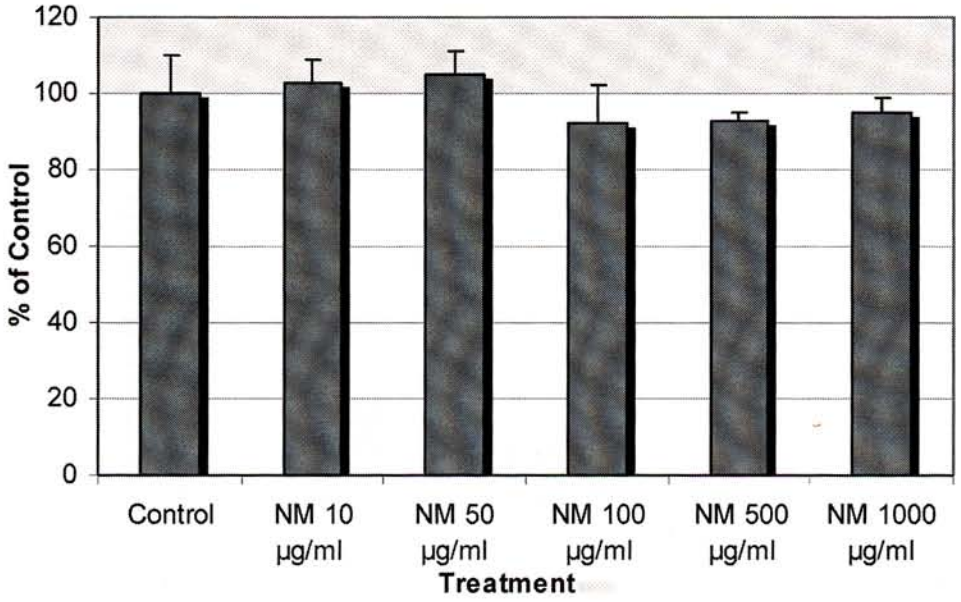

FIGURE 1 Effect of NM on growth of human malignant mesothelioma cell line MSTO-211-H at 24 hours: MTT assay.

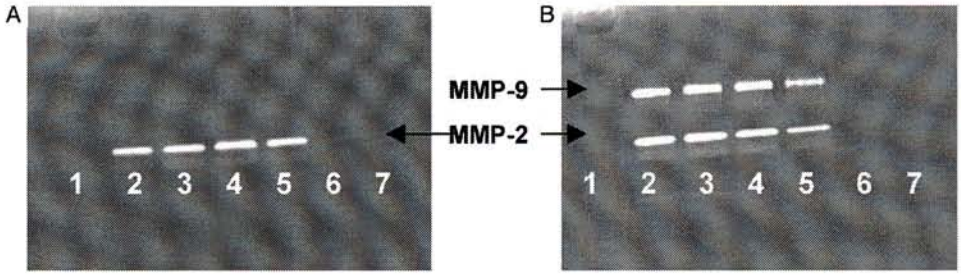

FIGURE 2 Effect of the nutrient mixture (NM) on malignant mesothelioma (MM) cell MSTO-211H MMP secretion. *A*, Untreated MM cells; *B*, PMA (200 ng/ml) treated MM cells. 1, markers; 2, control; 3–7, NM 10, 50, 100, 500, 1000 μg/ml.

of both MMPs in a dose-dependent fashion with virtual total inhibition at 500 μg/ml. See Figure 2*A*, *B*.

Invasion Study

The nutrient mixture (NM) significantly reduced the invasion of malignant mesothelioma cells through Matrigel in a dose-dependent fashion, with 27% inhibition at 50 μg/mL ($P = .002$), 36% inhibition at 100 μg/mL ($P = .009$), and 100% at 500 μg/mL ($P < .001$) NM, as shown in Figures 3 and 4.

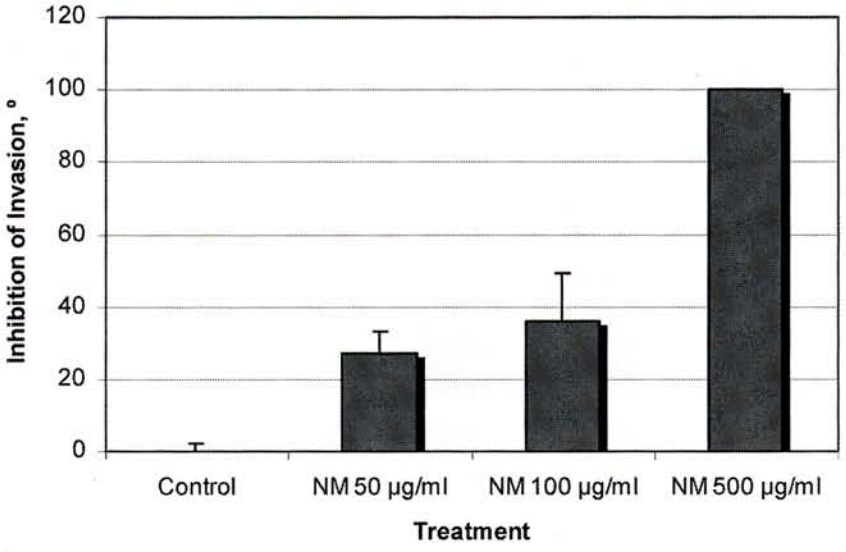

FIGURE 3 Effect of NM on human malignant mesothelioma cell MSTO-21H Matrigel invasion.

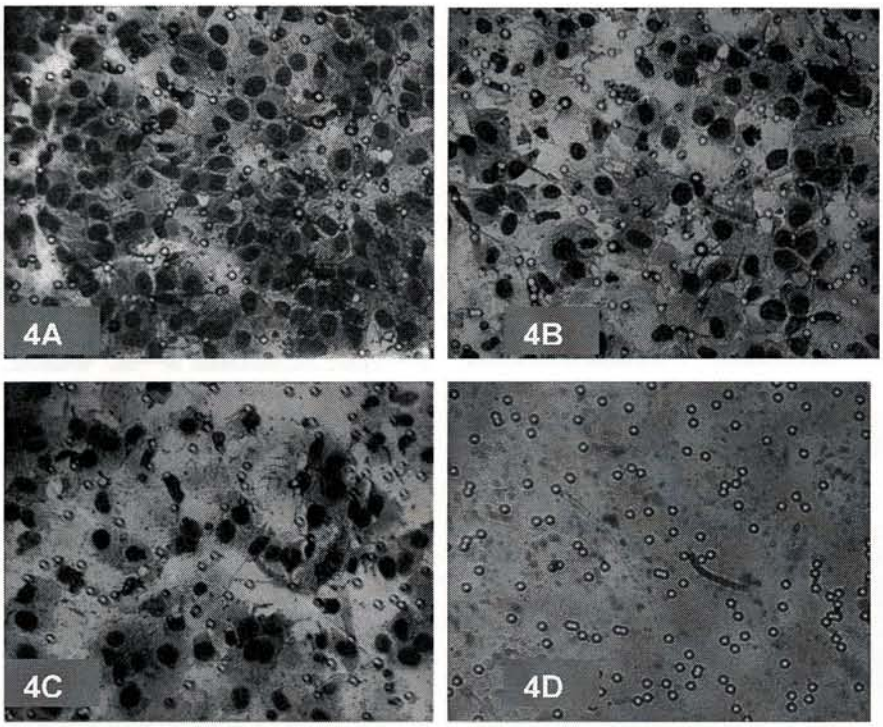

FIGURE 4 Invasion photomicrographs of human malignant mesothelioma cell MSTO-211H. *A*, Control; *B*, NM 50 μg/ml; *C*, NM 100 μg/ml; *D*, NM 500 μg/ml.

Morphology Study (Hematoxylin and Eosin Staining)

H&E staining showed no morphological changes at and below 100 μg/mL (Figure 5).

DISCUSSION

The nutrient mixture tested in this study was effective in complete inhibition of Matrigel invasion of human malignant mesothelioma MSTO-211H cells in vitro. Inhibition of Matrigel invasion was accompanied by a decrease in MMP-2 and MMP-9 secretion by mesothelioma cells in a dose-dependent fashion, with complete inhibition of both matrix invasion and MMP secretion at 500 μg/mL NM.

Clinical studies have shown that MMP expression is associated with malignant mesothelioma progression. In examining the expression of MMPs and collagens in 16 patients with pleural malignant mesothelioma, Hirano and colleagues [15] noted positive staining for MMP-1

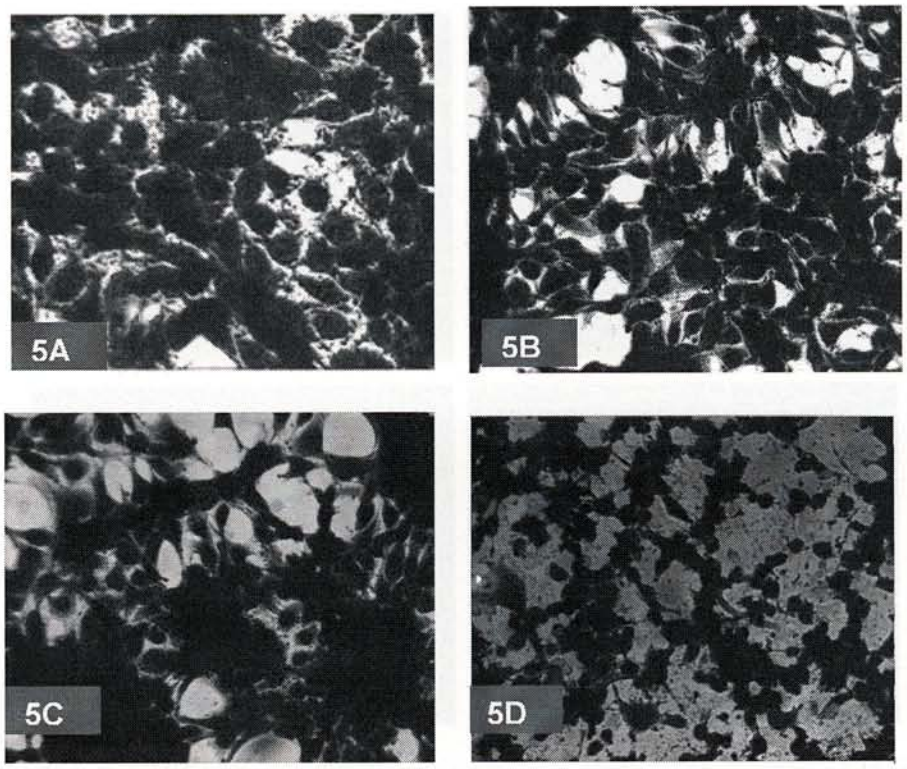

FIGURE 5 Effect of NM on morphology of human malignant mesothelioma cells MSTO-211H (H&E staining). *A*, Control; *B*, NM 50 μg/ml; *C*, NM 100 μg/ml; *D*, NM 500 μg/ml.

and MMP-2, concluding that expression of MMP-1 and MMP-2 may be related to pleural malignant mesothelioma invasion and spread. In a prospective study of gelatinase activity in homogenized supernatants of snap frozen malignant mesothelioma and inflamed pleura, pro- and active MMP-2 levels were significantly greater than MMP-9 in malignant mesothelioma and there was a trend towards poor survival with increasing total and proMMP-2 activity [16]. MMP-9 activity was not found to be prognostic. However, MMP activity is modulated by growth factors and MMP-9 expression by malignant mesothelioma cells appears to be induced by growth factors. In studying the effect of growth factors on MMP activity of human malignant mesothelioma cell lines in vitro, Liu and Klominek [17] observed that exposure of malignant mesothelioma cell SPC212 to different growth factors increased secretion of MMP-9 and MMP-3, but not MMP-2, and that β-cellulin and epidermal growth factor had the most potent effects on production of these MMPs. This induction of MMP production by different growth factors was inhibited

by the tyrosine kinase inhibitor genistein. In our study, NM was effective in inhibiting MMP activity in PMA-stimulated mesothelioma cells.

Progression of malignant tumors can be impeded through tumor-encapsulating effect by decreasing extracellular matrix degradation and optimizing its structure and integrity, thereby targeting universal pathomechanisms involved in cancer growth and invasion. Rath and Pauling [6] suggested targeting plasmin-mediated ECM degradation 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 tPA by binding to plasminogen active sites, and consequently prevents the plasmin-induced MMP activation cascade [6]. 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 [18].

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; in addition, low levels of ascorbic acid have been reported in cancer patients [19–24]. Green tea extract is a potent anticancer agent that has been reported to have a growth inhibitory effect against certain human cancer cell lines [25–27].

Furthermore, in contrast to chemotherapy agents, which causes indiscriminate cellular and ECM damage, our morphological studies of human malignant mesothelioma cells treated with NM showed that at NM concentrations below 100 μg/mL, these cells were not adversely affected. Furthermore, in a previous in vivo study addressing safety issues, we found that feeding the nutrient mixture (at 30, 90, or 150 mg per day for 7 days) to adult female ODS rats, weighing 250 to 300 g, 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 [28]. At the same time, the malignant cell growth and matrix invasion are significantly halted, consistent with the results of our previous study [14].

Although further studies on other mesothelioma cells lines are indicated to determine whether other mesothelioma cells would respond as the MSTO-211 line investigated in this study, the results of this study suggest that the formulation containing lysine, proline, arginine, ascorbic acid, and green tea extract tested is a promising candidate for therapeutic use in the treatment of mesothelioma, by inhibiting MMP secretion and invasion, and assuring safety at the cellular level.

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