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Original Article

Inhibition of Glioma Cell Line A-172 MMP Activity and Cell Invasion In Vitro by a Nutrient Mixture

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Abstract

Standard multimodality therapy of gliomas is associated with poor patient survival and significant toxicity. Abnormal expression of matrix metalloproteinases is associated with tumor growth and invasion. Based on reported antitumor properties, we investigated the effect of a combination of natural compounds (NM), primarily composed of lysine, proline, ascorbic acid, and green tea extract in vitro on glioma cell line A-172, by measuring MMP secretion, invasion through Matrigel, and cell proliferation. Glioma cells A-172 (ATCC) were grown in modified Dulbecco's Eagle medium with 10% fetal bovine serum and antibiotics and treated with NM at 0, 10, 50, 100, 500, and 1000 μg/mL concentration in triplicate at each dose. Cell proliferation was assayed by MTT, MMP secretion by zymography, invasion through Matrigel, and morphology by H&E staining. Zymography showed one band corresponding to MMP-2, which was inhibited by NM in a dose-dependent fashion, with virtual total inhibition at 500-μg/mL concentration. Invasion through Matrigel was completely inhibited at 1000 μg/mL NM. NM was not toxic to glioma cell line A-172 at lower concentrations and exhibited toxicity of 50% over the control at 1000 μg/mL. NM significantly inhibited MMP secretion and invasion-important parameters for cancer prevention, suggesting a possible therapeutic role.

Key Words: Glioma; MMPs; Matrigel invasion; nutrients.

Introduction

Approximately 18,820 new cases and 12,820 deaths from brain and other nervous system tumors are estimated in the United States for 2006, accounting for 2.4% of all cancer deaths. Brain tumors are the major cause of cancer-related death in patients under 15 yr of age, and cancer remains the leading medical cause of death among children in the United States *(1)*.

Roughly 40–50% of all pediatric CNS tumors are gliomas *(2)*. Despite advances, pediatric patient outcome remains poor with long-term survival rates ranging from 10% to 30% for supratentorial tumors *(3)* and less than 10% for diffuse brainstem gliomas *(4)*. At recurrence, two-thirds of supratentorial tumors are local, 10% are isolated leptomeningeal failures, and the remaining fall under a combination of both *(5)*.

Longer survival times have been achieved in patients with nondisseminated disease; however, adverse effects of surgical trauma and chemotherapeutic toxicity, such as subsequent malignancies have been widely reported *(6–9)*. Specifically, anthracyclines and mediastinal radiation are associated with congestive heart failure and cardiac impairment *(10,11)*. Both chemotherapy and radiation can result in pulmonary fibrosis, acute pulmonary toxicity, and restrictive lung disease *(12–14)*. Toxic impairment of hepatic, renal, and gastrointestinal systems by

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chemotherapeutic agents and radiation has also been reported *(15–17)*.

For the most part, cancer mortality is associated with distant metastases and consequent organ failure. In identifying the limitations and adverse effects of standard multimodality therapy, which fails to address metastasis, researchers have focused efforts on identifying various targets of cancer progression, including the inhibition of matrix metalloproteinases (MMPs). Key to metastasis and tumor growth is the degradation of components of the extracellular matrix (ECM) and tumor migration into nearby blood vessels. MMPs function to maintain balance between decomposition and restoration of tissue basic substance; however, when disturbances to this balance occur as with malignant disease, MMPs can support tumor cell invasion and metastasis by several mechanisms *(18)*. MMPs are capable of facilitating tumor cell invasion by degrading ECM macromolecules such as collagens, laminins, and proteoglycans. MMPs can also modulate cell adhesion and expose hidden biologic activities by acting on ECM components *(19–23)*. Studies have shown that increased expression of MMPs, especially MMP-2 and-9, correlate with poor patient survival and prognosis in a number of malignancies: breast cancer *(24)*, pancreatic cancer *(25)*, prostate cancer *(26)*, lung cancer *(27)*, esophageal cancer *(28)*, stomach cancer *(29)*, colon cancer *(30)*, ovarian cancer *(31)*, and brain cancer *(32)*, among others.

Therapies designed to prevent metastatic disease through inhibition of MMP show great promise in the treatment of cancer, especially aggressive highly invasive malignancies, resistant to standard combined approaches of surgery, radiotherapy, chemotherapy, and immunotherapy. In recognition of this, Rath and Pauling postulated that nutrients such as lysine and ascorbic acid (vitamin C) could modulate tumor growth and expansion by preventing ECM proteolysis and stabilizing connective tissue via inhibition of MMPs (preserving matrix integrity) and strengthening of connective tissue surrounding cancer cells (tumor-encapsulating effect) *(33)*. In previous studies, we demonstrated that the specific mixture of nutrients (NM), primarily composed of lysine, ascorbic acid, proline, and EGCG-enriched green tea extract, inhibited tumor growth, tissue invasion, angiogenesis, and MMP secretion in both animal model *(34)* and cell culture experiments *(35)*. In the current study, we investigated the anti-tumor potential of NM in vitro on glioma cell line A-172, by measuring MMP secretion, cell invasion through Matrigel, and cell proliferation.

Materials and Methods

Cell Culture

Glioma cells A-172 obtained from ATCC (American Type Culture Collection, Rockville, MD), were grown in Dulbecco's Eagle 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 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 1000 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. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) and 500 μL of MTT (Sigma cat. no. 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 secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymog-

raphy 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 37^2 C overnight in substrate buffer containing 50 m*M* Tris-HCl and 10 m*M* $CaCl_2$ 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 1200 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 to 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 glioma 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.

Morphology

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 nutrient mixture prepared for testing 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 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.

Statistical Analysis

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

Results

Cell Proliferation Study

NM was not toxic to glioma cell line A-172 at lower concentrations and exhibited toxicity of 50% over the control at 1000 μg/mL, $p = 0.003$, as shown in Fig. 1.

Gelatinase Zymography Study

Zymography demonstrated only one band corresponding to MMP-2. NM inhibited the secretion of MMP-2 in a dose-dependent fashion, with virtual inhibition at 500-μg/mL concentration, as shown in Fig. 2A. Densitometry analysis on relative activity of MMP-2 showed 21% inhibition at 50 μg/mL and 48% at 500 μg/mL and 99% at 1000 μg/mL, with $R^2 = 0.8514$ (Fig. 2B).

Invasion Study

The nutrient mixture (NM) significantly reduced the invasion of glioma cells through Matrigel in a dose-dependent fashion, with 93% inhibition at 500 μg/mL ($p = 0.003$) and 100% at 1000 μg/mL NM ($p = 0.002$), as shown in Figs. 3A–E.

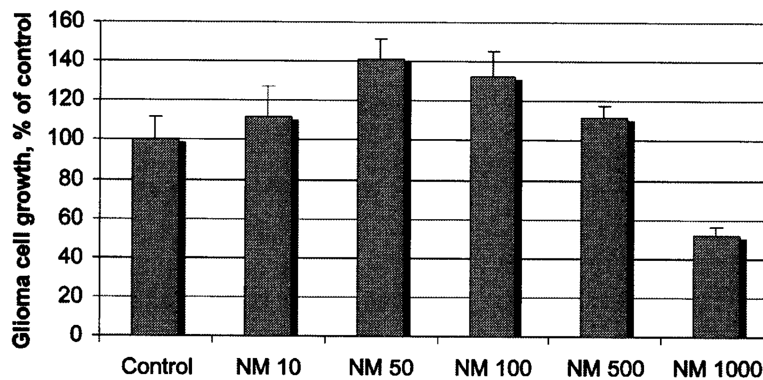

Fig. 1. Effect of the nutrient mixture (NM) on growth of glioma cells: 24 h MTT assay. NM was not toxic to glioma cell line A-172 at lower concentrations and exhibited toxicity of 50% over the control at 1000 μg/mL, $p = 0.003$

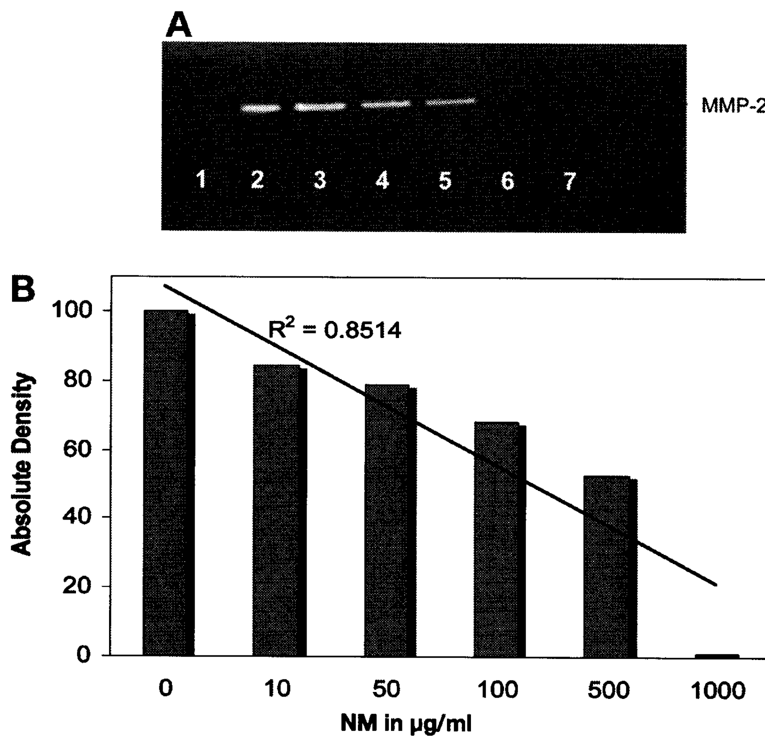

Fig. 2. Effect of the nutrient mixture (NM) on MMP-2 secretion by glioma cells. **(A)** Zymogram, Legend 1, Markers; 2 Control; 3–7 NM 10, 50, 100, 500, 1000 μg/mL. Zymography demonstrated only one band corresponding to MMP-2. NM inhibited the secretion of MMP-2 in a dose-dependent fashion, with virtual inhibition at 500-μg/mL concentration. **(B)** Densitometry analysis. Effect of nutrient mixture (NM) on relative activity of MMP-2 in human glioblastoma (A172).

Morphology Study (Hematoxylin and Eosin Staining)

H&E staining showed no morphological changes even at higher levels of NM (Figs. 4A–E).

Discussion

Glioblastoma demonstrates rapid progression and is highly resistant to standard treatment, resulting in a particularly poor prognosis in patients. Glioma-

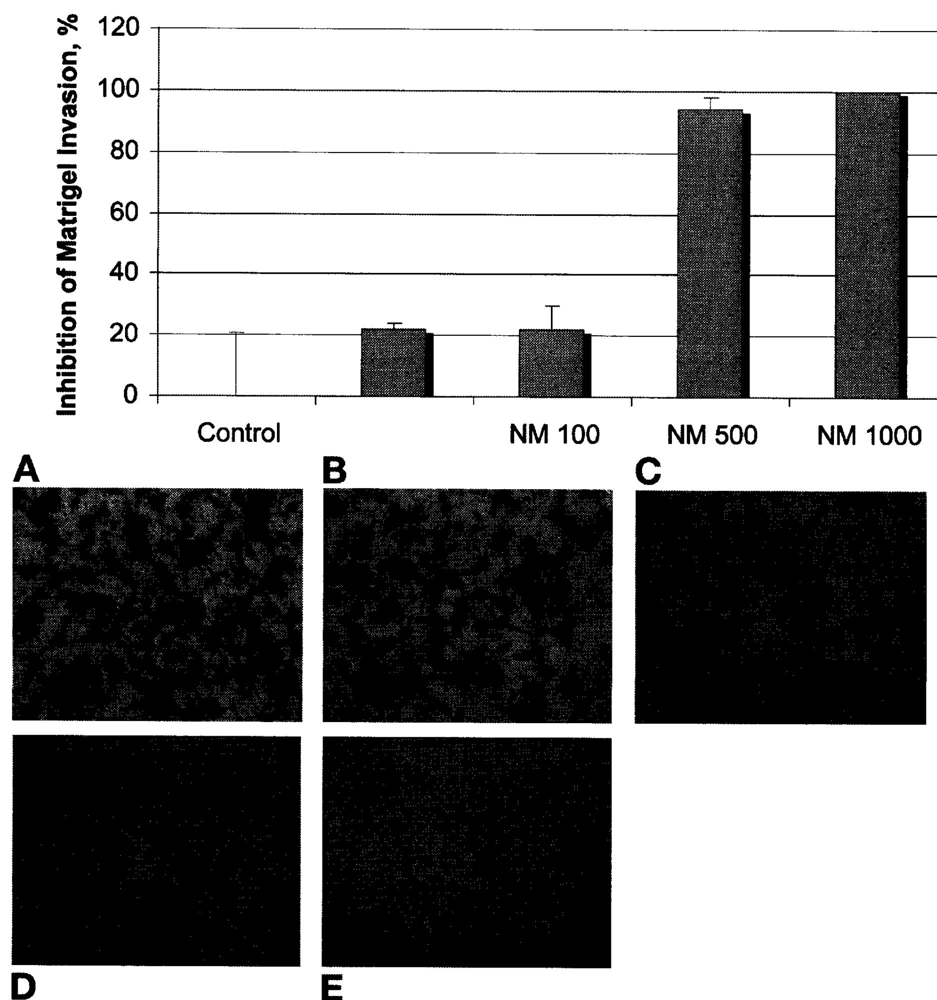

Fig. 3. Effect of the nutrient mixture (NM) on glioma cell Matrigel invasion. NM significantly reduced the invasion of glioma cells through Matrigel in a dose-dependent fashion, with 93% inhibition at 500 μg/mL (p = 0.003) and 100% at 1000 μg/mL NM (p = 0.002). (**A**) Control; (**B**) NM 50 μg/mL; (**C**) NM 100 μg/mL; (**D**) 500 μg/mL; (**E**) 1000 μg/mL.

blastoma is distinct from other cancer types by exhibiting profoundly vascularized tumors, necrosis, and microvascular hyperplasia. Pseudopalisades and microvascular hyperplasia are indicative of aggressive growth and are instrumental in malignant progression *(36)*. Pseudopalisading cells also express high levels of vascular endothelial growth factor (VEGF), which promote endothelial proliferation and angiogenesis. The nutrient mixture (NM) tested was formulated based on targeting different physiological processes involved in cancer progression and metastasis. Selenium, a component of NM, has been shown to prevent migration of endothelial cells through the ECM *(37)*. Green tea extract, another major component in NM, has been shown to control angiogenesis by inhibition of angiogenic factors such as VEGF *(38)*. In addition, we recently showed that NM caused a significant (p < 0.05) reduction in bFGF-induced angiogenesis in a chorioallantoic membrane (CAM) assay in chick embryos, as well as decreased human osteosarcoma U2OS cell expression of VEGF, angiopoietin-2, bFGF, PDGF, and TGFbeta-1 *(39)*.

Additionally, increased MMP levels are associated with poor prognosis in gliomablastoma patients. MMP-2 can cleave laminin 5 $\gamma 2$ and release proteolytic fragments which are capable of leading to