In vitro and in vivo effects of a nutrient mixture on breast cancer progression

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Abstract. Long-term survival of patients with breast cancer remains poor, due to metastasis and recurrence. We investigated the effects of a novel nutrient mixture (NM) containing ascorbic acid, lysine, proline and green tea extract in vitro and in vivo on 4T1 murine breast cancer, a representative model for metastatic breast cancer. After one week of isolation, 5-6-week-old female Balb/C mice were inoculated with 5x10^4 4T1 cells into the mammary pad and randomly divided into two groups; the control group was fed a regular diet and the NM group a regular diet supplemented with 0.5% NM. After four weeks, the mice were sacrificed and their tumors, lungs, livers, kidneys, hearts and spleens were excised and processed for histology. Dimensions (length and width) of tumors were measured using a digital caliper, and the tumor burden was calculated using the following formula: 0.5 x length x width. We also tested the effect of NM in vitro on 4T1 cells, measuring cell proliferation by MTT assay, MMP secretion by zymography, invasion through Matrigel, migration by scratch test and morphology by H&E staining. NM inhibited tumor weight and burden of 4T1 tumors by 50% (p=0.02) and 53.4% (p≤0.0001), respectively. Lung metastasis was profoundly inhibited by NM supplementation: mean number of colonies was reduced by 87% (p<0.0001) and mean weight of lungs by 60% (p=0.0001) compared to control mice. Metastasis to liver, spleen, kidney and heart was significantly reduced with NM supplementation. In vitro, NM exhibited 50% toxicity over the control at 250 and 500 µg/ml concentrations. Zymography demonstrated MMP-2 and MMP-9 secretion which was inhibited by NM in a dose-dependent manner, with virtual total inhibition of both at 1,000 µg/ml. Migration by scratch test and invasion through Matrigel were inhibited in a dose-dependent manner with total block of invasion at 250 and of migration at 1,000 µg/ml. These results suggest that NM has therapeutic potential in the treatment of breast cancer.

Introduction

Breast cancer, the most prevalent cancer in women worldwide and the leading cause of cancer death in women, was projected to claim the lives of ~39,620 women in the USA in 2013 (1). Though treatable in early stages, once metastasis has occurred, the survival rate is drastically reduced to a median of 2-3 years and treatment focuses on palliative care (2).

Critical events in tumor cell invasion include cell attachment, proteolytic degradation of the extracellular matrix (ECM) and migration through the disrupted matrix (3). Rath and Pauling (4) proposed that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM degradation, inhibiting MMP activity and strengthening the connective tissue surrounding cancer cells, and thus potentially modulating tumor growth and expansion. We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid and green tea extract (NM). This nutrient mixture has exhibited synergistic anticancer activity in vivo and in vitro in a number of cancer cell lines through inhibition of cancer cell growth, MMP secretion, invasion, metastasis and angiogenesis (5).

A major problem in studying metastasis has been the lack of suitable models that faithfully represent the metastatic process as it occurs in vivo. While some human xenograft models can approximate primary tumor growth in mice, replication of tumor metastasis is more problematic (6-8). Generally, human tumor cells metastasize poorly in mice and metastases are associated with unexpected characteristics. In contrast, murine tumor cell models often metastasize more effectively and display metastatic characteristics more similar to those observed in cancer patients (9). Since microenvironments and tumor-host interactions play important roles in tumor cell behavior, this is not surprising. When introduced orthotopically, 4T1 is capable of metastasis to several organs affected in breast cancer, including lungs, liver and brain as well as bone.

In this study, our main objective was to determine the effect of dietary supplementation with NM on the development of tumors and metastasis to other organs challenging mice with breast cancer 4T1 cells into the mammary pad. The 4T1 mammary carcinoma model was chosen as it has several characteristics that make it a suitable experimental animal model for human mammary cancer growth and metastasis (10,11). The tumor cells are easily transplanted into the mammary gland so that the primary tumor grows in the mammary pad and treatment focuses on palliative care (2).
the anatomically correct site and, as in human breast cancer,
4T1 metastatic disease develops spontaneously from the
primary tumor. In addition, metastatic spread of 4T1 meta-
stases to other organs and the draining lymph nodes is similar
to that of human mammary cancer (10). In addition, we studied
the effect of NM on 4T1 cells in vitro evaluating viability,
MMP secretion, migration and invasion.

Materials and methods

Cancer cell line and culture. Murine breast cancer cell line 4T1
was obtained from ATCC (American Type Culture Collection,
Rockville, MD, USA). 4T1 cells were maintained in DMEM,
supplemented with 10% fetal bovine serum, 100 U/ml peni-
cillin and 100 µg/ml streptomycin. The media and sera used
were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL (Long Island, NY, USA).

Composition of the nutrient mixture. The nutrient mixture
(NM) 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 1,000 mg; L-proline 750 mg;
L-arginine 500 mg; N-acetyl cysteyne 200 mg; standardized
green tea extract (derived from green tea leaves, was obtained
from US Pharma Lab; the certificate of analysis indicated the
following characteristics: total polyphenol 80%, catechins 60%,
epigallocatechin gallate (EGCG) 35% and caffeine 1.0%];
1,000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.

In vivo studies

Animals. Female Balb/C mice, approximately five weeks of
age on arrival, were purchased from Simonsen Laboratories
(Gilroy, CA, USA) and maintained in microisolator cages under
pathogen-free conditions on a 12-h light/12-h dark schedule for
a week. All procedures were performed according to humane
and customary care and use of experimental animals and
followed a protocol approved by internal institutional animal
safety review committee.

Experimental design. After housing for a week, the mice
(n=14) were inoculated with 5x10^5 4T1 cells in 0.2 ml PBS
and 0.1 ml Matrigel (BD Bioscience, Bedford, MA, USA) into
the mammary pad. After injection, the mice were randomly
divided into two groups and maintained for four weeks on the
following diets; the control group mice were fed regular Purina
mouse chow and the NM group the regular diet supplemented with
0.5% NM (w/w). During the study, the mice consumed,
for 30 min at room temperature to remove SDS. The gels
were then 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 absorb-
bance 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. Gelatinase zymography was
performed in 10% Novex Pre-Cast SDS polyacrylamide
gel (Invitrogen Corp.) in the presence of 0.1% gelatin under
non-reducing conditions. Culture media (20 µl) were mixed
with sample buffer and loaded for SDS-PAGE with tris glycine
SDS buffer, as suggested by the manufacturer (Novex). Samples
were not boiled before electrophoresis. Following electropho-
resis 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, 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. Upon
renaturation of the enzyme, the gelatinases digested the gelatin
in the gel, producing clear bands against an intensely stained
background. Protein standards were run concurrently and
approximate molecular weights were determined by plotting
the relative mobilities of known proteins.

Migration: scratch test. To study cell migration, a 2-mm wide
single uninterrupted scratch was made from top to bottom of
culture plates of cancer cells grown to confluence. Culture
plates were washed with PBS and incubated with NM in
medium and tested at 0, 50, 100, 250, 500 and 1,000 µg/ml in
triplicate at each dose for 24 h. Cells were washed with PBS, fixed and stained with H&E and photomicrographs were taken.

**Matrigel invasion.** Invasion studies were conducted using Matrigel (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, 4T1 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 a 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.

**Statistical analysis.** The results are expressed as means ± SD, as indicated in the results, for the groups. Data was analyzed by independent sample *t*-test.

**Results**

**In vivo studies**

**Tumor weight and burden.** NM strongly inhibited tumor growth and burden of 4T1 tumors in female Balb/C mice. Mean tumor weight was inhibited by 50% (p=0.02) with NM 0.5% dietary supplementation, as shown in Fig. 1A and tumor burden was inhibited by 53.4% (p<0.0001), as shown in Fig. 1B. Mean tumor weight of supplemented mice was 0.91±0.43 g and that of mice on the control diet 1.83±0.81 g. Mean tumor burden of supplemented mice was 140±48 cm$^2$ and that of mice on the control diet 300±45 cm$^2$. Images of mice and gross tumors from groups are shown in Fig. 2.

**Tumor histopathology.** Histologically, both groups demonstrated irregularly round subcutaneous tumors with large central areas of tumor necrosis involving 70% of the tumor.
Figure 3. Tumor histopathology. (A) Control tumor, x40; (B) control tumor, x200; (C) NM 0.5% tumor, x40; (D) NM 0.5% tumor, x200.

Figure 4. Lung metastasis. (A) Mean number of colonies in lungs of control and NM 0.5% group; (B) comparative weights of lungs in control and NM 0.5% group (the asterisk indicates significance of p=0.0001 with respect to control).
mass in the control mice and 50-70% in the supplemented mice. Viable, peripheral tumor tissue consisted of sheaths of small irregularly-round to spindle-shaped cells with poorly defined cytoplasm and often vesiculated nuclei. Mitotic figures averaged 1-2 per high-power field (Fig. 3).

Metastasis to lungs. Mice supplemented with NM 0.5% showed profoundly reduced number of colonies in the lungs as contrasted to the lungs of control mice. Mean number of colonies in the lungs of supplemented mice (9±8.4) were 13% (p<0.0001) of the mean number of colonies in the lungs of control mice (69±15.6), as shown in Fig. 4A. Furthermore, mean weight of lungs of supplemented mice (0.24±0.05 g) were 40.7% (p=0.0001) of the mean weight of lungs of control mice (0.59±0.16 g), as shown in Fig. 4B. Images of gross lungs from groups are shown in Fig. 5.

Lung histopathology. Multiple metastases were observed in the lungs of control mice in contrast to few, small metastatic lesions in lungs of NM supplemented mice. Neoplastic cells were large, irregularly round, with prominent large, irregularly round nuclei and scant cytoplasm (Fig. 6).

Mean weights of livers, kidneys, spleens and hearts. No significant differences were found between control and NM supplemented mean organ weights, as shown in Fig. 7.

Metastasis to liver. Two of three liver sections examined from control group livers showed 2-3 small, metastatic lesions associated with severe, perivascular and sinusoidal neutrophilic infiltration. The third section had no evidence of metastasis or severe neutrophilic infiltration, but did have multifocal areas of severe, acute liver necrosis. All four sections of liver examined in NM 0.5% fed mice, showed no definite metastatic lesions. Many vessels were severely cuffed with neutrophils. A
few questionable cells presented in sinusoids, but these most likely were myeloid in origin. Gross images of control livers are shown in Fig. 8 and histopathology of livers from control and supplemented mice is shown in Fig. 9.

Metastasis to kidney. Three partial kidney sections of control mice presented subcapsular, metastatic lesions and one section areas of acute infarction. Of three sections of NM kidney examined, no metastases or specific changes were noted. Gross images of control kidneys are shown in Fig. 10 and histopathology of kidneys from control and supplemented mice is shown in Fig. 11.

Metastasis to heart. Among the control group heart sections examined, four of five showed myocardial metastatic lesions, one large and three smaller metastases. In the NM heart sections examined, two of four sections each had a metastatic lesion near the base of the heart. Gross images of hearts from both groups are shown in Fig. 12 and histopathology of hearts from control and supplemented mice is shown in Fig. 13.

Metastasis to spleen. Of the three sections of control group spleens examined, all showed severe, extramedullary hematopoiesis and 2-3 small metastases. Sections of NM spleen showed severe, extramedullary hematopoietic activity and a small, metastatic lesion in one section. Gross images of control spleens are shown in Fig. 14 and histopathology of spleens from control and supplemented mice is shown in Fig. 15.

In vitro

Cell proliferation: MTT assay. NM exhibited dose-dependent inhibition of 4T1 cell growth with 50% (p<0.0001) antiproliferative effect at 250 and 500 µg/ml and 60% (p<0.0001) at 1,000 µg/ml compared to control, as shown in Fig. 16.
Figure 11. Histopathology of kidneys. (A) Kidney control, x100; (B) kidney NM 0.5%, x100.

Figure 12. Gross images of hearts.

Figure 13. Histopathology of hearts. (A) Control heart, x40; (B) NM 0.5% heart, x40.

Figure 14. Gross images of control spleen.
Gelatinase zymography. Zymography demonstrated MMP-2 and MMP-9 secretion by untreated and PMA-treated 4T1 cells. NM inhibited secretion of both MMPs in a dose-dependent manner with virtual total inhibition of both at 1,000 µg/ml, as shown in Fig. 17.

Migration: scratch test. NM reduced breast cancer 4T1 cell migration in a dose-dependent manner, with total inhibition at 1,000 µg/ml, as shown in Fig. 18.

Matrigel invasion. NM significantly inhibited 4T1 invasion through Matrigel in a dose-dependent manner, with total block at 250 µg/ml, as shown in Fig. 19.

Morphology: H&E staining. H&E staining showed no morphological changes even at higher concentrations of NM, as shown in Fig. 20.

Discussion

The results of the in vivo study of murine 4T1 cells injected into the mammary pads of Balb/C mice demonstrated significant suppression of tumor growth (50% reduction in tumor weight and 53% in tumor burden) with NM dietary supplementation. Lung metastasis was profoundly inhibited by NM supplementation: mean number of colonies was reduced by 87% and mean weight of lungs by 60% compared to control mice. Metastasis to liver, spleen, kidney and heart was significantly reduced with NM supplementation. The results from the in vitro studies support the in vivo findings. In vitro, NM inhibited cell proliferation by 50% at 250 and 500 µg/ml concentrations compared to the control. Zymography demonstrated MMP-2 and MMP-9 secretion which was inhibited by NM in a dose-dependent manner, with virtual total inhibition.
of both at 1,000 µg/ml. Migration by scratch test and invasion through Matrigel were inhibited dose-dependently with total block of invasion at 250 and of migration at 1,000 µg/ml.

Tao et al reported that metastasis of 4T1 tumors was associated with extensive necrosis and inflammation within the primary tumor and hematopoiesis in several mouse organs including spleen and liver (10). In our study, both groups demonstrated irregularly round subcutaneous tumors with large central areas of tumor necrosis involving 70% of the

Figure 18. Effect of NM on 4T1 cell migration: scratch test. (A) Pre-scratch; (B) control 24 h; (C) NM 50 µg/ml 24 h; (D) NM 100 µg/ml 24 h; (E) NM 250 µg/ml 24 h; (F) NM 500 µg/ml 24 h; (G) NM 1,000 µg/ml 24 h.
tumor mass in the control mice and 50-70% in the supplemented mice. As discussed earlier, we observed multiple metastases in the lungs of control mice in contrast to few small metastatic lesions in lungs of NM supplemented mice. Splenic metastasis showed severe, extramedullary hematopoiesis and 2-3 small metastases in the spleen sections of control mice and a small metastatic lesion and extramedullary hematopoietic activity in the sections of supplemented mice. Liver metastasis in our study showed several small metastatic lesions associated with severe, perivascular and sinusoidal neutrophilic infiltration in the control sections and absence of metastatic lesions but presence of neutrophils in supplemented sections.

Bonfil et al. reported that the distribution of necrosis within a primary tumor was responsible in part for the development of metastases (12), and that tumor necrosis was an important source of gelatinase/type IV collagenase, mainly in its 92-kDa form, and thus played a major role in tumor invasion (13). In a previous study, we found that among gulo KO mice injected with mammary 4T1 cells, those mice deprived of ascorbate developed large tumors with dark cores, showing more necrosis, and poorly defined borders, while gulo KO mice supplemented with ascorbate hosted smaller tumors with smaller, lighter cores, less necrosis and enhanced collagen encapsulation, signifying less metastatic potential (14).

High MMP-2 and MMP-9 levels have been found to correlate with aggressiveness of cancers, as exemplified by breast cancer (15,16). Our present in vitro study showed dose-dependent inhibition of MMP-2 and MMP-9 secretion, cell migration and cell invasion through Matrigel with treatment of 4T1 cells with NM.

A causal relationship between inflammation and 4T1 metastasis was proposed by Connolly et al. based on observation of an inhibitory effect of the COX-2 inhibitor sC-236 on metastasis of 4T1 after primary tumor excision (17). Inflammation has been noted to have a positive effect on metastasis in several systems (18,19). The NM has been shown to have an inhibitory effect on inflammatory mediators as COX-2 in prior studies (5,20,21).

Optimal ECM structure depends upon adequate supplies of ascorbic acid and the amino acids lysine and proline to ensure proper synthesis and hydroxylation of collagen fibers. In addition, lysine contributes to ECM stability as a natural inhibitor of plasmin-induced proteolysis (4,22). Manganese and copper are also essential for collagen formation. There is considerable documentation of the potency of green tea extract in modulating cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (23-27). N-acetyl cysteine and selenium have demonstrated inhibition of tumor cell MMP-9 and invasive activities, as well as migration of endothelial cells through ECM (28-30). Ascorbic acid demonstrates cytotoxic and antimetastatic actions on malignant cell lines (14,31-35) and cancer patients have been found to have

Figure 19. Effect of NM on 4T1 cell invasion through Matrigel; photomicrographs. (A) Control; (B) NM 50 µg/ml; (C) NM 100 µg/ml; (D) NM 250 µg/ml.
low levels of ascorbic acid (36,37). Low levels of arginine, a precursor of nitric oxide (NO), can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis (38).

In conclusion, the results of the in vivo study of murine 4T1 cells injected into the mammary pads of Balb/C mice confirmed the validity of this model to study breast cancer metastasis since metastasis was observed in lung, liver, spleen, kidney and heart, as in human breast cancer. The present study also demonstrated significant suppression of tumor growth and metastasis to all of these organs with NM dietary supplementation, which indicates NM inhibition of metastasis is based on targeting common parameters as MMPs and the collagen barrier. Furthermore, the in vivo results were supported by in vitro findings of NM suppression of cell proliferation, secretion of MMP-2 and MMP-9, migration and Matrigel invasion by 4T1 cells. These results suggest that NM has therapeutic potential in treatment of breast cancer.
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References