In vitro modulation of MMP-2 and MMP-9 in human cervical and ovarian cancer cell lines by cytokines, inducers and inhibitors

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Abstract. Matrix metalloproteinases (MMPs) secreted by cervical and ovarian cancer, especially MMP-2 and MMP-9, play crucial roles in tumor invasion and metastasis. We examined the effect of cytokines, mitogens, inducers and inhibitors on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines. Human cervical (HeLa and DoTc2-4510) and ovarian (SK-OV-3) cell lines were cultured in appropriate media. At near confluence, the cells were washed with PBS and incubated in serum-free medium with various concentrations of several cytokines, mitogens and inhibitors. After 24 h the media were removed and analyzed for MMP-2 and MMP-9 by gelatinase zymography and quantitated by densitometry. HeLa and SK-OV-3 cell lines expressed MMP-2 whereas DoTc2-4510 cells expressed MMP-9. Treatment of cervical cancer cell lines (HeLa and DoTc2-4510) with PMA had no effect on MMP-2 expression and a moderate stimulatory effect in ovarian cancer cell line SK-OV-3. MMP-9 was stimulated by phorbol 12-myristate 13-acetate in HeLa cells and enhanced in DoTc2-4510. Tumor necrosis factor-α and interleukin-1ß, had slight inhibitory effect on HeLa cell expression of MMP-2 while lipopolysaccharide stimulated MMP-2 in HeLa cells. Doxycycline, epigallocatechin gallate, a nutrient mixture, actinomycin-D, cyclohexamide, retinoic acid and dexamethasone inhibited MMP-2 in HeLa and SK-OV-3 cell lines and inhibited MMP-9 in DoTc2-4510. Our results show that cytokines, mitogens, inducers and inhibitors have an up or down regulatory effect on MMP-2 and MMP-9 expression in ovarian and cervical cancer cell lines, suggesting these agents may be effective strategies to treat these cancers.

Introduction

Cervical cancer is the fifth most common malignant neoplasm worldwide and the second most common in less developed countries (1). The death rate for cervical cancer, which was once one of the most common causes of cancer death for American women, declined by 74% between 1955 and 1992, mainly due to the increased use of the Pap test (2). The American Cancer Society estimates that in 2009, ~11,270 cases of invasive cervical cancer will be diagnosed in the United States and ~4,070 women will die from cervical cancer (2). Cervical cancer develops slowly, taking 10-15 years to develop into cancer, starting with a pre-cancerous condition called dysplasia that can be detected by pap smears and is fully treatable. However, once the cancer has metastasized, patient outcome is poor. Epithelial ovarian carcinoma, which occurs mainly in post-menopausal women, is the leading cause of death from gynecological malignancy and the fifth most common cancer in the US (3). Since ovarian cancer often remains clinically silent, the majority of patients with ovarian carcinoma have advanced intraperitoneal metastatic disease at diagnosis, resulting in a poor prognosis. About 80% of ovarian cancer cases are diagnosed at an advanced stage after metastasis has occurred (3). Matrix metalloproteinase (MMP) expression has been shown to be linked to tumor invasion in many different tumors (4,5-10). In addition, clinical studies note the association of MMP expression with progression of cervical (11,12) and ovarian (13,14) cancer.

MMPs degrade various components of the extracellular matrix (ECM), including collagen, laminin, fibronectin, vitronectin, elastin and proteoglycans. Since MMPs play a critical role in cancer invasion, migration metastasis and tumorigenesis, blocking tumor cell expression of MMPs can significantly reduce tumor invasion and metastasis (5,6,15). Although there are five major classes of proteases, interest has been focused on type IV collagenases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), both of which cleave collagen IV, a major component of ECM and basement membrane. Most MMPs are secreted as zymogens and must be activated extracellularly. MMP-2 (72 kDa) and MMP-9 (92 kDa) are secreted in their latent zymogenic form and cleaved by other MMPs or proteases to yield the activated forms of 68, 58 and 54 kDa for MMP-2 and 94 kDa for MMP-9. Many human tumors have been reported to be associated with increased expression of MMP-2 and MMP-9 (16-18) and tumor aggression has been found to significantly correlate with increased levels of MMP-2 and MMP-9 in many experimental and clinical studies (5-10).

MMP activity is regulated by, and dependent upon environmental influences from surrounding stroma, ECM proteins, systemic hormones and other factors (17,19,20). A

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Table I. Quantitative densitometry results from the effects of inducers PMA, TNF-α, IL-1ß and LPS on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines.

	Cervical cancer (HeLa)		Cervical cancer (DoTc2-4510)		Ovarian cancer (SK-OV-3)	
	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)
PMA (ng/ml)						
Control	12.6	0.0	0	10.4	17.7	0
10	12.1	10.5	0	19.4	22.3	0
25	12.2	10.4	0	23.9	20.2	0
50	12.2	10.3	0	24.3	19.1	0
100	10.7	9.1	0	22.0	20.7	0
TNF-α (ng/ml)						
Control	15.9	0.0	0	12.9	19.2	0
0.1	15.3	11.1	0	17.9	21.8	0
1	15.0	14.2	0	19.1	25.9	0
10	15.4	0.0	0	22.2	17.4	0
25	13.1	0.0	0	27.9	15.7	0
IL-1ß (ng/ml)						
Control	25.3	0	0	17.3	19.3	0
0.1	21.0	0	0	20.7	20.3	0
1	21.1	0	0	26.5	18.6	0
10	18.3		0	24.6	19.4	0
25	14.2	0	0	10.9	22.3	0
LPS (μg/ml)						
Control	15.3	0	0	15.0	20.5	0
10	16.1	1.7	0	20.3	18.5	0
25	16.8	2.0	0	22.3	21.1	0
50	20.3	2.2	0	21.4	20.3	0
100	23.4	2.3	0	21.0	19.6	0

variety of cytokines and growth factors, such as transforming growth factor (TGF-ß), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and tumor necrosis factor (TNF-α) also control MMP activity (21,22). One of the most potent inducers is the chemical agent phorbol 12-myristate 13-aceteate (PMA). In addition, activity of MMPs is regulated at multiple levels, including transcription, modulation of messenger RNA half-life (translation), secretion, localization, activation and inhibition (23).

In this study, we investigated the roles of cytokines, inducers and inhibitors in the regulation of MMP-2 and MMP-9 activities in two related female cancers, cervical and ovarian cancer.

Materials and methods

Materials. Human cervical cancer cell lines HeLa and DoTc2-4510 and ovarian cancer cell line SK-OV-3, along with their culture media MEM and McCoy, respectively, were obtained from ATCC. Antibiotics, penicillin and fetal bovine serum (FBS), were obtained from Gibco (BRL, Long Island, NY). Twenty-four-well tissue culture plates were obtained from Costar (Cambrdige, MA). Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen Inc.) with 0.1% gelatin in non-reducing conditions. Interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), PMA, lipopolysaccharide (LPS), doxycycline, epigallocatechin gallate (EGCG), cyclohexamide, actinomycin-D, retinoic acid and dexamethasone, were purchased from Sigma (St. Louis, MO). The nutrient mixture (NM), prepared by VitaTech (Hayward, CA) was composed of the following ingredients in the relative amounts 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 (80% polyphenol) 1000 mg; selenium 30 μg; copper 2 mg; manganese 1 mg. All other reagents used were of high quality and were obtained from Sigma, unless otherwise indicated.

Cell cultures. Human cervical cancer cell lines HeLa and DoTc2-4510 were grown in MEM and ovarian cancer cell line SK-OV-3 in McCoy medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 24-well tissue culture plates. The cells were plated at a density of $1x10^5$ cells/ml and maintained in a humidified atmosphere

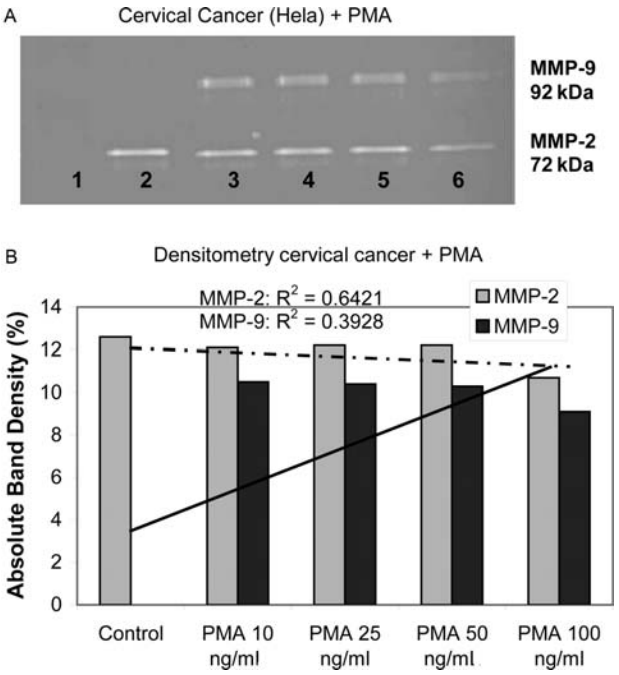

Figure 1. Effect of PMA on MMP expression in cervical cancer cell line HeLa. (A) Gelatinase zymogram of HeLa cells showing induction of MMP-9 expression after PMA treatment. Untreated HeLa cells expressed only MMP-2. PMA induced MMP-9 in a dose-dependent manner but had no effect on MMP-2 expression. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 ng/ml PMA. (B) Quantitative densitometric analysis of MMP expression of HeLa gelatinase zymogram. MMP expression is reported relative to the band density of MMP-2 in the control lane. Trend analysis: R^2=0.6421 MMP-2; R^2=0.3928 MMP-9.

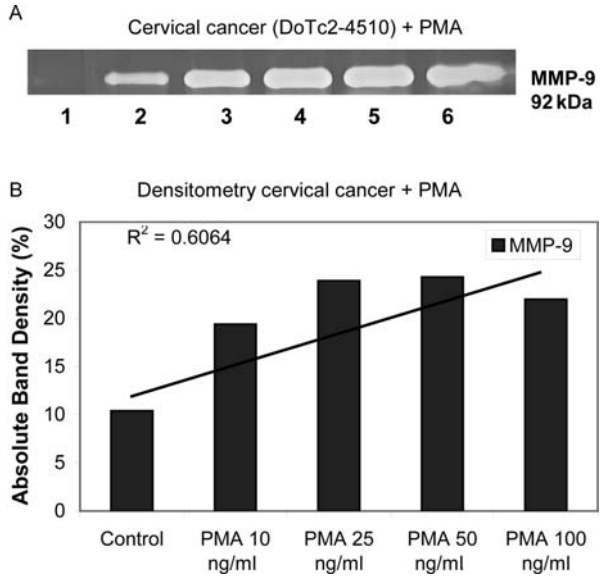

Figure 2. Effect of PMA treatment on MMP expression in cervical cancer cell line DoTc2-4510. (A) Gelatinase zymogram of DoTc2-4510 cells showing dose-dependent stimulation of MMP-9 expression with PMA treatment. DoTc2-4510 cells only expressed MMP-9. MMP-2 expression was not induced with PMA treatment. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 ng/ml PMA. (B) Quantitative densitometric analysis of MMP expression of DoTc2-4510 gelatinase zymogram. MMP expression is reported relative to the band density of MMP-9 in the control lane. Trend analysis: R^2=0.6064.

of 5% CO_2 in air at 37˚C until confluent. Serum-supplemented medium was removed and cell monolayer was washed twice with PBS and once with serum-free media. The cells were then incubated in 0.5 ml of serum-free medium with various cytokines, mitogens, inducers and inhibitors in triplicates, as indicated: PMA (10, 25, 50, 100 ng/ml); TNF-α (0.1, 1, 10, 25 ng/ml); IL-ß (0.1, 1, 10, 25 ng/ml); LPS (10, 25, 50, 100 μg/ml); EGCG (10, 25, 50, 100 μM) without and with PMA; doxycycline (10, 25, 50, 100 μM) without and with PMA; NM (10, 50, 100, 500, 1000 μg/ml) without and with PMA; retinoic acid (50 μM); dexamethasone (50 μM); actinomycin-D (2 and 4 μg/ml); and cyclohexamide (2 and 4 μg/ml). The plates were then returned the to the incubator. The conditioned medium from each treatment was collected separately, pooled, and centrifuged at 4˚C for 10 min at 3,000 rpm to remove cells and cell debris. The clear supernatant was collected and used for gelatinase zymography, as described below.

Gelatinase zymography. Gelatinase zymography was utilized because of its high sensitivity to gelatinolytic enzymatic activity and ability to detect both pro and active forms of MMP-2 and MMP-9. Upon renaturation of the enzyme, the gelatinases digest the gelatin in the gel and reveal clear bands against an intensely stained background. Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel 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 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 $CaCl_2$ 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 300 dpi. The intensity of the bands was evaluated using the 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).

Results

Effect of inducers PMA, TNF-α, IL-1ß and LPS on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines. Table I shows the quantitative densitometry results from the effects of inducers PMA, TNF-α, IL-1ß and LPS on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines (HeLa, DoTc2-4510 and SK-OV-3). As shown in Fig. 1A and B, PMA had no significant effect on HeLa cell expression of MMP-2, but stimulated MMP-9 expression in a dose-dependent manner (10-100 ng/ml). DoTc2-4510 showed no MMP-2 expression with or without PMA; however, expression of MMP-9 was stimulated with PMA treatment, as shown in Fig. 2A and B. SK-OV-3 showed

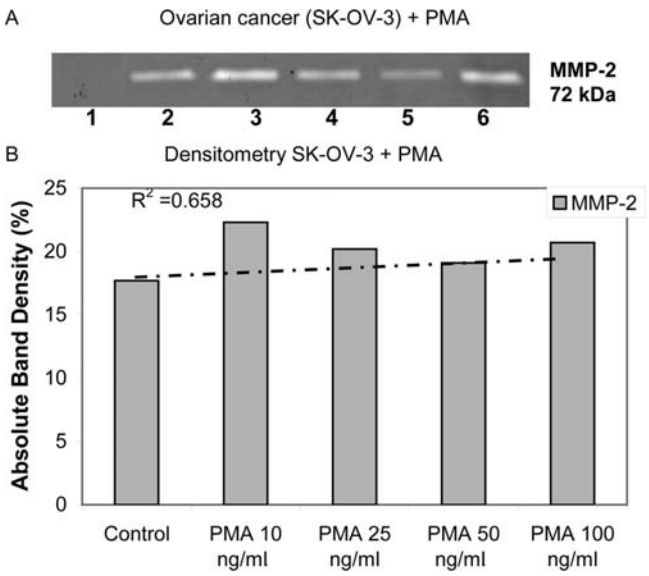

Figure 3. Effect of PMA treatment on MMP expression in ovarian cell line SK-OV-3. (A) Gelatinase zymogram of SK-OV-3 cells showing expression of MMP-2, which was neither induced nor inhibited by PMA treatment. PMA treatment did not induce MMP-9 expression. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 ng/ml PMA. (B) Quantitative densitometric analysis of MMP expression of SK-OV-3 gelatinase zymogram. MMP expression is reported relative to the band density of MMP-2 in the control lane. Trend analysis: R^2=0.658.

a single band corresponding to MMP-2, which was not affected by PMA treatment (Fig. 3A and B). TNF-α had a stimulatory dose-dependent effect on MMP-9 in DoTc2-4510, did not affect MMP-2 expression in HeLa cells, and slightly stimulated MMP-2 expression in ovarian cancer cells SK-OV-3. IL-1ß stimulated MMP-9 in DoTc2-4510 cells at 0.1 and 1 ng/ml, but had an inhibitory effect at 10 and 25 ng/ml. IL-1ß had a dose-dependent inhibitory effect on MMP-2 in HeLa cells and no effect in SK-OV-3 cells. LPS showed a dose-dependent enhanced effect on MMP-2 and MMP-9 expression in HeLa cells and MMP-9 in DoTc2-4510, but no effect on MMP-2 in ovarian cancer cells.

Effect of inhibitors doxycycline and EGCG on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines. Table II shows the quantitative densitometry results from the effects of inhibitors doxycycline and EGCG on MMP-2 and MMP-9 expression in untreated and PMA-treated cervical and ovarian cancer cell lines (HeLa, DoTc2-4510 and SK-OV-3). Doxycycline inhibited the expression of MMP-2 in HeLa (Fig. 4A and B) and SK-OV-3 cells (Fig. 6A and B), and the expression of MMP-9 in DoTc2-4510 (Fig. 5A and B) in a dose-dependent fashion. HeLa cells were most sensitive to doxycycline, followed by DoTc2-4510 and SK-OV-3. PMA-treated HeLa cells exposed to doxycycline (Fig. 4C and D) showed lower MMP-2 activity than cells not treated with PMA and dose-dependent inhibition with increasing doxycycline dose. PMA induced MMP-9 expression by HeLa cells that was inhibited in a dose-dependent manner with increased doxycycline. Doxycycline inhibited PMA-induced MMP-9 expression in DoTc2-4510 cells (Fig. 5C and D) and MMP-2 expression in SK-OV-3 cell lines (Fig. 6C and D) in a dose-dependent manner. Doxycycline inhibited the

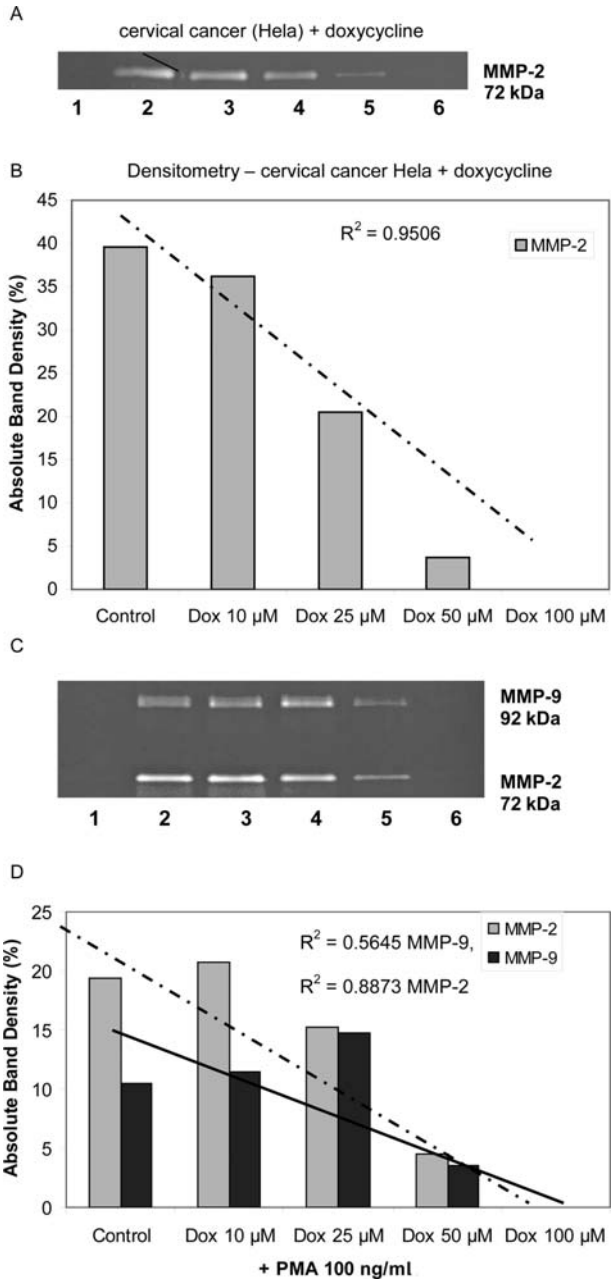

Figure 4. Effect of doxycycline on MMP expression in PMA (100 ng/ml)-treated cervical cancer cell line HeLa. (A) Gelatinase zymogram of HeLa cells showing dose-dependent decrease of MMP-2 expression with doxycycline treatment. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (B) Quantitative densitometric analysis of MMP expression in doxycycline-treated HeLa cells. Trend analysis R^2=0.9506. (C) Gelatinase zymogram of HeLa cells stimulated to express MMP-9 via PMA (100 ng/ml) showing a dose-dependent decrease of both MMP-2 and MMP-9 expression with increasing concentrations of doxycycline. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (D) Quantitative densitometric analysis of MMP expression in PMA-stimulated HeLa cells treated with doxycycline. MMP expression is reported relative to the band density of MMP-2 in the control lane. Trend analysis: R^2=0.5645 MMP-9; R^2=0.8873 MMP-2.

expression of MMP-2 in HeLa and SK-OV-3 cell lines equally. EGCG decreased both MMP-2 and MMP-9 expression in HeLa cells and completely abolished their expression at 50 μM concentration. EGCG decreased MMP-9 expression in DoTc2-4510 and MMP-2 in ovarian cancer cell line SK-OV-3. Sensitivity to EGCG was greater in DoTc2-4510 cells, followed by HeLa and SK-OV-3 cells.

Table II. Quantitative densitometry results from the effects of inhibitors doxycycline and EGCG on MMP-2 and MMP-9 expression in untreated and PMA-treated cervical and ovarian cancer cell lines.

	Cervical cancer (HeLa)		Cervical cancer (DoTc2-4510)		Ovarian cancer (SK-OV-3)	
	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)
Doxycycline (μM)						
Control	39.6	0	0	24.7	25	0
10	36.2	0	0	22.9	22	0
25	20.5	0	0	20.8	27	0
50	3.7	0	0	18.9	16	0
100	0.0	0	0	12.7	9	0
Doxycycline (μM) with PMA (200 ng/ml)						
Control	19.4	10.5	0	52.6	29.1	0
10	20.7	11.5	0	30.0	26.6	0
25	15.2	14.8	0	15.2	21.6	0
50	4.5	3.5	0	2.2	17.2	0
100	0.0	0.0	0	0.0	5.5	0
EGCG (μM)						
Control	39.0	0	0	78.4	38.0	0
10	33.9	0	0	14.9	21.4	0
25	27.1	0	0	4.2	19.8	0
50	0.0	0	0	2.5	16.5	0
100	0.0	0	0	0.0	4.3	0
EGCG (μM) with PMA (200 ng/ml)						
Control	35.9	20.0	0	45.9	32.4	0
10	11.1	15.1	0	35.4	20.9	0
25	5.1	10.8	0	15.4	13.2	0
50	0.9	1.1	0	3.2	19.5	0
100	0.0	0.0	0	0.2	14.1	0
Dexamethasone (μM)						
Control	62.7	0	0	100	82.5	0
100	37.3	0	0	0	17.5	0
Actinomycin-D (μg/ml)						
Control	63.0	0	0	52.4	36.5	0
2	19.4	0	0	27.9	34.7	0
4	17.6	0	0	19.7	28.8	0
Cyclohexamide (μg/ml)						
Control	100	0	0	100	87.0	0
2	0	0	0	0	7.7	0
4	0	0	0	0	5.3	0
Retinoic Acid (μM)						
Control	x	x	0	100	89.0	0
50	x	x	0	0	11.0	0

Effect of inhibitors retinoic acid, dexamethasone, actinomycin-D and cyclohexamide, on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines. Table II shows the quantitative densitometry results from the effects of inhibitors retinoic acid, dexamethasone, actinomycin-D and cyclohexamide on MMP-2 and MMP-9 expression in untreated and PMA-treated cervical and ovarian cancer cell lines (HeLa, DoTc2-4510, and SK-OV-3). Dexamethasone at

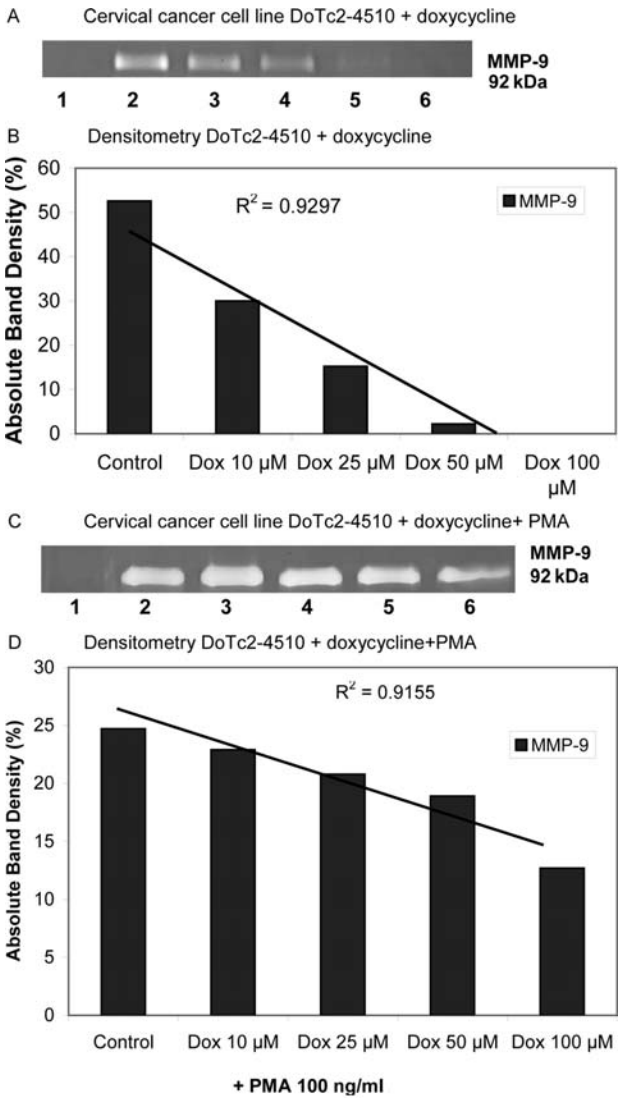

Figure 5. Effect of doxycycline on MMP expression in PMA-treated cervical cancer cell line DoTc2-4510. (A) Gelatinase zymogram of DoTc2-4510 cells showing a dose-dependent decrease of MMP-9 expression with doxycycline treatment. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (B) Quantitative densitometric analysis of MMP-9 expression in doxycycline-treated DoTc2-4510 cells. MMP expression is reported relative to the band density of MMP-9 in the control lane. Trend analysis: R^2=0.9155. (C) Gelatinase zymogram of DoTc2-4510 cells showing enhanced MMP-9 expression via PMA (100 ng/ml) treatment and dose-dependent decrease of MMP-9 expression with increasing concentrations of doxycycline. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (D) Quantitative densitometric analysis of MMP-9 expression in PMA stimulated DoTc2-4510 cells treated with doxycycline. Trend analysis: R^2=0.9297.

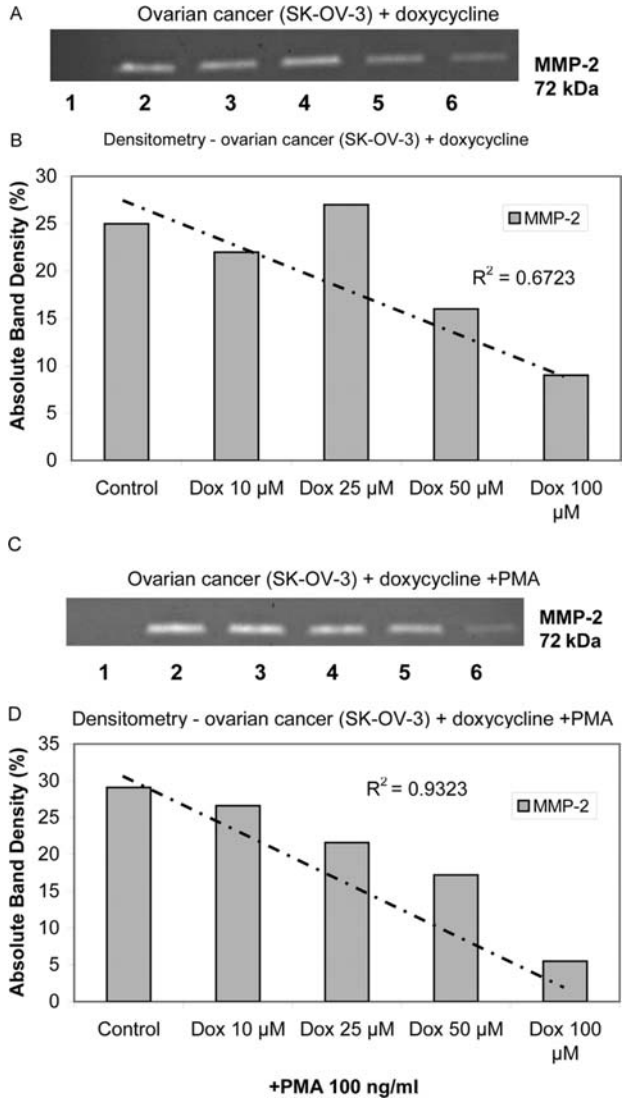

Figure 6. Effect of doxycycline on MMP expression in PMA-treated and untreated ovarian cancer cell line SK-OV-3. (A) Gelatinase zymogram of SK-OV-3 cells showing dose-dependent decrease of MMP-2 expression with doxycycline treatment. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (B) Quantitative densitometric analysis of MMP-2 expression in doxycycline treated SK-OV-3 cells. Trend analysis: R^2=0.6723. (C) Gelatinase zymogram of PMA-treated (100 ng/ml) SK-OV-3 cells showing no effect of PMA on the dose-dependent decrease of MMP-2 expression via doxycycline treatment. 1, Makers; 2, Control; 3-6, 10, 25, 50, 100 μM doxycycline. (D) Quantitative densitometric analysis of MMP-2 expression in SK-OV-3 cells treated with PMA and doxycycline. Trend analysis: R^2=0.9323.

100 μM inhibited the expression of MMP-2 in HeLa cells by 40% of control level and completely blocked MMP-9 expression in DoTc2-4510 cells. In SK-OV-3, MMP-2 expression was decreased by 79% of the control at a concentration of 100 μM dexamethasone. Retinoic acid, on the other hand, abolished MMP-9 activity in DoTc2-4510 and reduced MMP-9 expression by 12% in SK-OV-3. Actinomycin-D at 4 μg/ml inhibited the expression of MMP-2 in HeLa to 28% of control, MMP-9 in DoTc2-4510 to 38% of control and MMP-2 in ovarian cancer cell line SK-OV-3 to 79% of control. Cyclohexamide, at concentrations 2 and 4 μg/ml completely abolished the expression of MMP-2 in HeLa cells, MMP-9 in DoTc2-4510 cells and MMP-2 to 10 and 6% of the control level in SK-OV-3. Retinoic acid at 50 μM abolished MMP-9 expression by DoTc2-4510 cells and inhibited MMP-2 expression of SK-OV-3 cells to 12% of the control value.

Effect of the nutrient mixture (NM) on MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines. As shown in Table III and Figs. 7-9, NM had a dose-dependent inhibitory effect on all the cell lines. NM inhibited MMP-2 expression in HeLa cells in unstimulated conditioned media (Fig. 7A and B) and MMP-2 and MMP-9 in PMA-treated

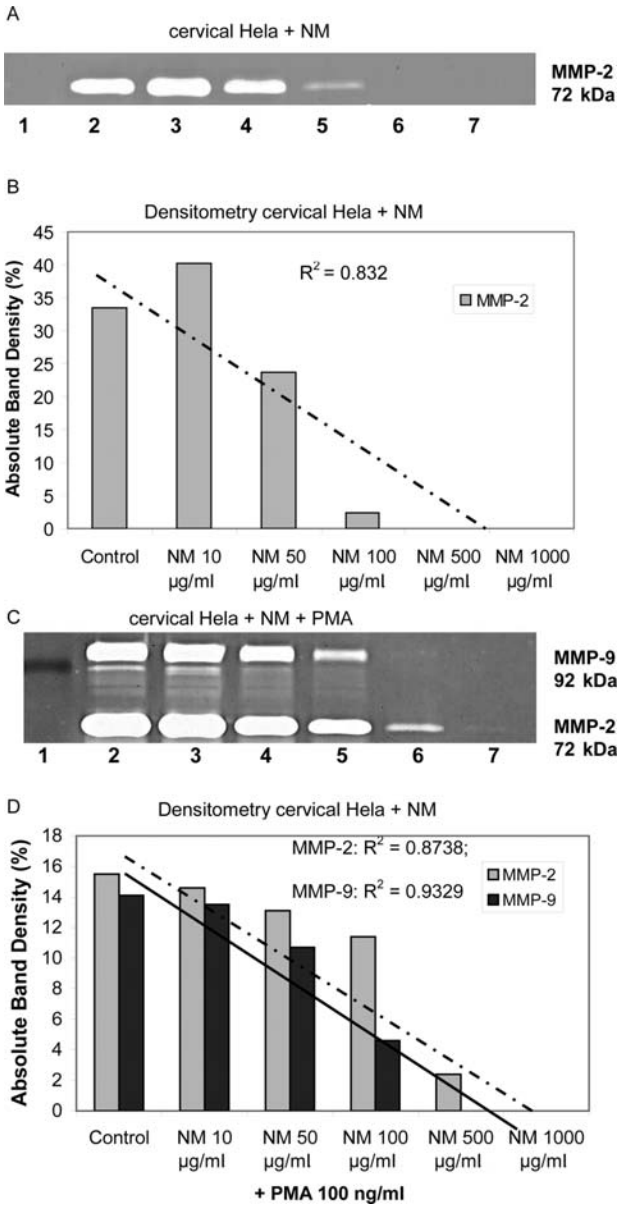

Figure 7. Effect of the nutrient mixture on MMP expression in PMA-treated and untreated cervical cell Line HeLa. (A) Gelatinase zymogram showing dose-dependent decrease of MMP-2 expression by HeLa cells with NM treatment. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (B) Quantitative densitometric analysis of MMP-2 expression by HeLa cells treated with NM. Trend analysis: R^2=0.832. (C) Gelatinase zymogram of PMA (100 ng/ml)-treated HeLa cells showing induction of MMP-9 but no effect on MMP-2 expression and dose-dependent decrease in MMP-2 and MMP-9 expression with NM treatment. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (D) Quantitative densitometric analysis of MMP-2 and MMP-9 expression in HeLa cells treated with PMA and the nutrient mixture. Trend analysis: R^2=0.8738 MMP-2; R^2=0.9329 MMP-9.

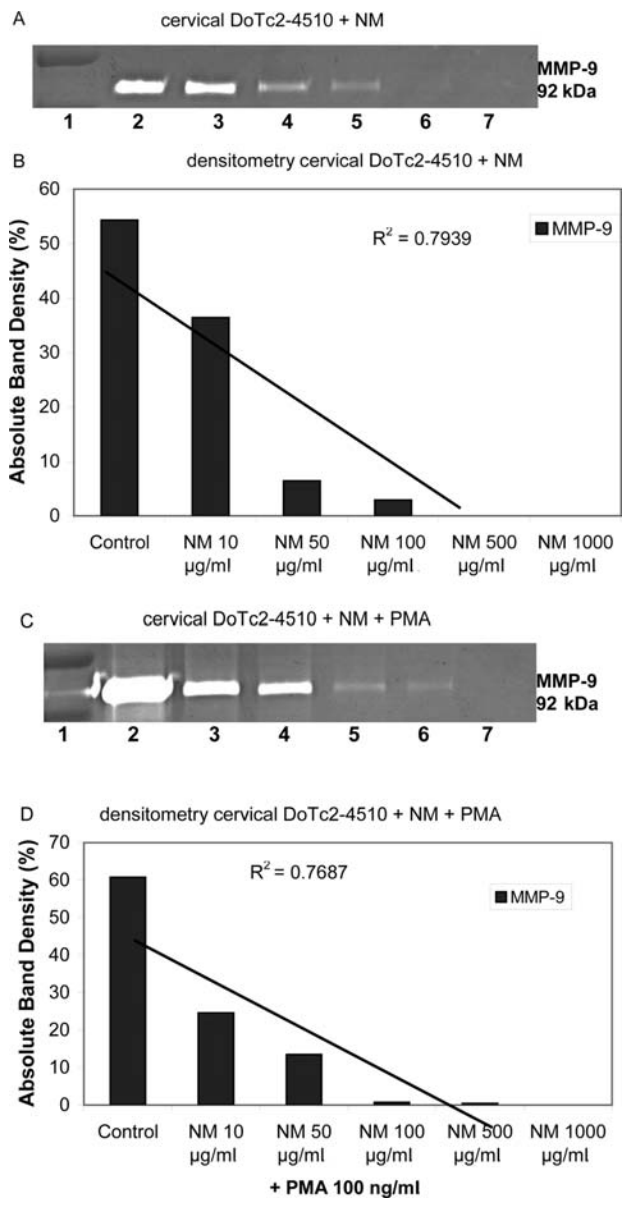

Figure 8. Effect of the nutrient mixture on MMP expression in PMA-treated and untreated cervical cell line DoTc2-4510. (A) Gelatinase zymogram of DoTc2-4510 cells showing dose-dependent decrease of MMP-9 expression with NM treatment. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (B) Quantitative densitometric analysis of MMP-9 expression in DoTc2-4510 cells treated with NM. Trend analysis: R^2=0.7939. (C) Gelatinase zymogram of PMA (100 ng/ml)-treated DoTc2-4510 cells showing enhanced MMP-9 expression with PMA and dose-dependent decrease of MMP-9 with increasing concentration of NM. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (D) Quantitative densitometric analysis of MMP-9 expression in DoTc2-4510 cells treated with PMA and NM. Trend analysis: R^2=0.7687.

conditioned media (Fig. 7C and D) in a dose-dependent fashion, with complete blockage of MMP expression at 500 μg/ml. DoTc2-4510 showed MMP-9 expression (Fig. 8A and B), which was further enhanced by PMA (Fig. 8C and D); NM inhibited MMP-9 in a dose-dependent fashion, virtually abolishing MMP-9 at 500 μg/ml. Ovarian cancer cell MMP-2 expression was unaffected by PMA treatment; NM inhibited MMP-2 expression in a dose-dependent manner with total blockage at 50 μg/ml NM (Fig. 9A and D).

Discussion

Experimental and clinical studies have shown a correlation between increased MMPs and tumor progression and metastasis (5-10). Thus, knowledge of MMP regulation is of great importance for developing therapeutic strategies. MMP expression is regulated at both pre and post-transcriptional levels. A number of extracellular factors, including cytokines, growth factors, cell contact with ECM, and inducers and

Table III. The dose-dependent inhibitory effect of the nutrient mixture on the cell lines.

	Cervical cancer (HeLa)		Cervical cancer (DoTc2-4510)		Ovarian cancer (SK-OV-3)	
	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)
Nutrient mixture (NM) (μg/ml)						
Control	33.5	0.0	0	54.3	70.9	0
10	40.2	0.0	0	36.4	29.1	0
50	23.6	0.0	0	6.4	0.0	0
100	2.5	0.0	0	2.9	0.0	0
500	0.0	0.0	0	0.0	0.0	0
1000	0.0	0.0	0	0.0	0.0	0
Nutrient mixture (NM) (μg/ml) with PMA (200 ng/ml)						
Control	15.5	14.1	0	60.8	71.8	0
10	14.6	13.5	0	24.6	28.2	0
50	13.1	10.7	0	13.5	0.0	0
100	11.4	4.6	0	0.7	0.0	0
500	2.4	0.0	0	0.4	0.0	0
1000	0.0	0.0	0	0.0	0.0	0

inhibitors, have been implicated in the regulation of MMP expression in different types of tumor cells (24,25).

In this study, we compared MMP secretion patterns by cytokines, PMA and LPS in two related human cancers, cervical and ovarian cancer. In addition, we investigated the effect of inhibitors doxycycline and EGCG and others, such as dexamethasone, retinoic acid and agents that affect transcription and translation levels, such as actinomycin-D and cyclohexamide. Furthermore, we tested a nutrition mixture that had inhibitory effects on MMP-2 and MMP-9 expression. Normal and cancerous ovarian and cervical cells produce immunomodulatory cytokines; however cytokine expression patterns change after malignant transformation (26,27). We chose cervical and ovarian cancer cell lines that express MMP-2 and MMP-9 to different extents. MMP-9 mRNA and protein expression have been shown to be significantly elevated in tumor and stromal cells of invasive squamous cell carcinoma of the uterine cervix (28). MMP-9 was also reported to be significantly associated with nodal metastasis of cervical cancer (13). Of interest, Torng *et al* report that though tumor MMP-2 levels are associated with poor prognosis of ovarian cancer, stromal MMP-2 levels in epithelial ovarian carcinoma were significantly associated with recurrence-free survival (14).

We found that cervical cancer cell line HeLa and ovarian cancer cell line SK-OV-3 normally expressed only MMP-2, whereas DoTc2-4510 normally expressed only MMP-9. Treatment of cervical cancer cell lines (HeLa and DoTc2-4510) with PMA had no effect on MMP-2 expression; however, MMP-2 was slightly stimulated by PMA in SK-OV-3 cells. MMP-9 was stimulated by PMA in HeLa cells at 10 ng/ml and this upregulated level of expression was maintained with increasing doses of PMA. PMA also enhanced MMP-9 expression in DoTc2-4510. However, MMP-9 expression by SK-OV-3 was not induced by any of the inducers tested. TNF-α had no effect on MMP-2 expression in HeLa cells, but slightly stimulated MMP-9 expression in HeLa cells and had a dose-dependent stimulatory effect on MMP-9 in DoTc2-4510. TNF-α had a slight stimulatory effect on MMP-2 secretion by SK-OV-3 cells at lower concentrations (0.1-1 ng/ml) and inhibition at 10-25 ng/ml. IL-1ß had an inhibitory effect in HeLa cell MMP-2 expression and a stimulatory effect on MMP-9 in DoTc2-4510. LPS had a stimulatory effect on both MMP-2 and MMP-9 in HeLa cells and enhanced MMP-9 expression in DoTc2-4510 cells. However, IL-1ß and LPS had no significant effect on MMP-2 secretion by SK-OV-3 cells.

Doxycycline and EGCG, without and with PMA, inhibited the expression of MMP-2 and MMP-9 expression in a dose-dependent fashion in HeLa cells, whereas MMP-9 was inhibited in DoTc2-4510 and MMP-2 in SK-OV-3. Sensitivity of inhibitors (decreasing magnitude of MMP-2 and MMP-9 expression) occurred in the following manner: DoTc2-4510>HeLa>SK-OV-3. In contrast, sensitivity to actinomycin-D was found to follow a different sequence: HeLa>DoTc2-4510>SK-OV-3. The nutrition mixture, without and with PMA, decreased MMP-2 and MMP-9 expression in a dose-dependent fashion in HeLa cells and induced a significant decrease of MMP-9 expression in DoTc2-4510. It exhibited a much higher inhibitory effect on MMP-2 expression by SK-OV-3 than by HeLa or inhibition of MMP-9 in DoTc2-4510.

The nutrition mixture studied, which contains lysine, proline, ascorbic acid and green tea extract among other micronutrients, has been shown to have anti-tumor and anti-invasive potential *in vivo* (29) and *in vitro* (30). In this study, NM, without and with PMA, showed a dose-dependent decrease of MMP-2 and MMP-9 in the cancer cell lines tested.

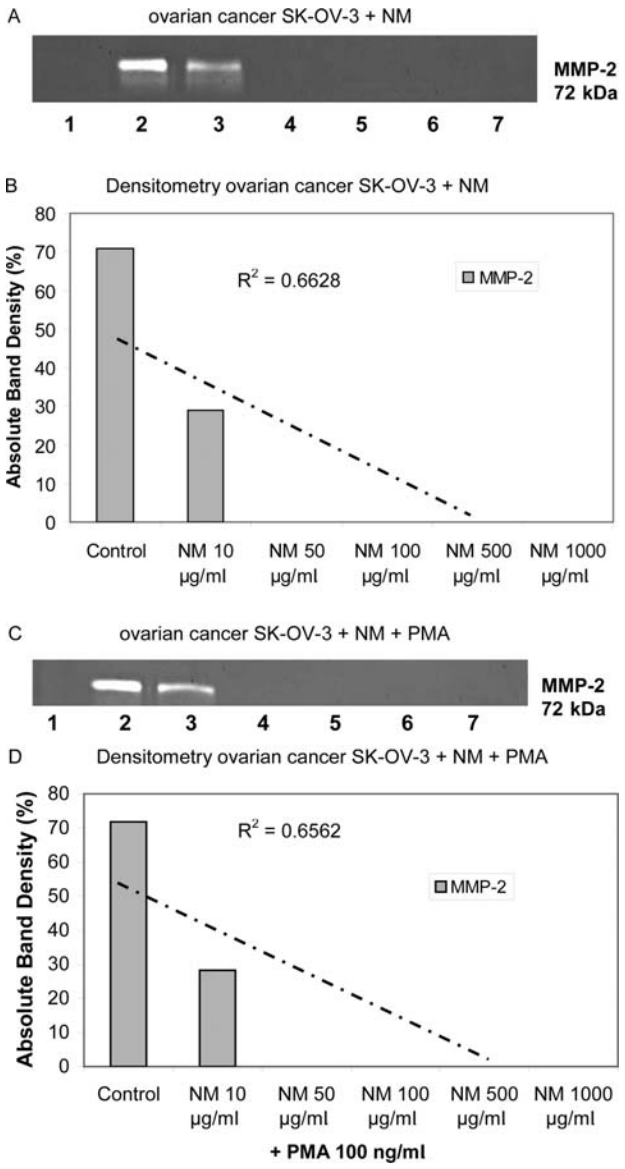

Figure 9. Effect of the nutrient mixture on MMP expression in PMA-treated and untreated ovarian cell line SK-OV-3. (A) Gelatinase zymogram of SK-OV-3 cells showing dose-dependent decrease of MMP-2 expression with NM treatment. MMP-2 expression is completely inhibited at 50 μg/ml NM. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (B) Quantitative densitometric analysis of MMP-2 expression in SK-OV-3 cells treated with NM. Trend analysis: R^2=0.6628. (C) Gelatinase zymogram of PMA-treated SK-OV-3 cells showing dose-dependent decrease of MMP-2 with increasing NM concentration. 1, Makers; 2, Control; 3-7, 10, 50, 100, 500, 1000 μg/ml NM. (D) Quantitative densitometric analysis of MMP-2 expression in SK-OV-3 cells treated with PMA and the nutrient mixture. Trend analysis: R^2=0.6562.

Of interest, in previous studies we found that NM had slight anti-proliferative effect on ovarian cancer cells SK-OV-3 cells but significant dose-dependent inhibition of Matrigel invasion of SK-OV-3 cells (31). In HeLa and DoTc2 4510 cells, NM had significant anti-proliferative effect as well as significant inhibition of Matrigel invasion (30).

We designed NM by focusing on physiological targets in cancer progression and metastasis, such as ECM integrity and MMP activity. Adequate supplies of ascorbic acid, lysine and proline to ensure proper synthesis and hydroxylation of collagen fibers are required for optimal ECM formation and structure. Manganese and copper are also essential for collagen formation. Lysine, a natural inhibitor of plasmin-induced proteolysis, contributes to ECM stability (32,33). Green tea extract has been shown to control cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (34-38). N-acetyl cysteine and selenium have been observed to inhibit MMP-9 activity and tumor invasion (39-41). Ascorbic acid was shown to inhibit cell division and growth through production of hydrogen peroxide (42). Arginine, a precursor of nitric oxide (NO), has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells (43).

In conclusion, our results show that cytokines and inhibitors regulate MMP-2 and MMP-9 expression in cervical and ovarian cancer cell lines HeLa, DoTc2-4510, and SK-OV-3, suggesting the clinical value of targeting these proteases for management of cervical and ovarian cancers and their pathogenesis.

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