Modulation of MMP-2 and -9 secretion by cytokines, inducers and inhibitors in human melanoma A-2058 cells

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Abstract. Melanoma, an extremely aggressive cancer, causes the most skin cancer-related deaths, due to metastasis to other areas of the body, such as lymph nodes, lungs, liver, brain or bone. It is characterized by high levels of matrix metalloproteinase (MMP)-2 and -9 secretions that degrade the extracellular matrix and basement membrane, allowing cancer cells to spread to distal organs. Various cytokines, mitogens, growth factors, inducers and inhibitors control MMP activities. We investigated the roles of these in regulation of MMP-2 and -9 in human melanoma A-2058 cells. Human A-2058 cells were grown in DMEM supplemented with 15% FBS and antibiotics in 24-well tissue culture plates. At near confluence, the cells were washed with PBS and incubated in serum-free media with phorbol 12-myristate 13-acetate (PMA) at 10, 25, 50 and 100 ng/ml; TNF-α and IL-1β at 0.1, 1, 10 and 25 ng/ml; LPS at 10, 25, 50 and 100 μg/ml; epigallocatechin gallate (EGCG) and doxycycline (Dox) at 10, 25, 50 and 100 μM without and with PMA; a nutrient mixture (NM) containing lysine, proline, ascorbic acid and green tea extract without and with PMA at 10, 50, 100, 500 and 1,000 μg/ml; actinomycin D and cyclohexamide at 2 and 4 μM; retinoic acid and dexamethasone at 50 μM. After 24 h the media were removed and analyzed for MMP-2 and MMP-9 by zymography and densitometry. Melanoma A-2058 demonstrated strong expression of MMP-2 and slight expression of MMP-9. PMA at 100 ng/ml showed no effect on MMP-2 secretion but potently upregulated MMP-9 secretion to 400% that of control. TNF-α showed no significant overall effect on expression of MMP-2 but potent dose-dependent increased MMP-9 secretion with 200% of control at 25 ng/ml. IL-1β showed no significant effect on MMP-2 or MMP-9 secretion by A-2058 cells, except at 25 ng/ml where MMP-2 level was reduced by ~40% and MMP-9 secretion ~50%. LPS treatment showed no significant effect on MMP-2 secretion and enhanced MMP-9 secretion up to 25 μg/ml followed by decreased level. EGCG, NM and doxycycline, without and with PMA, downregulated the expression of MMP-2 and MMP-9 in a dose-dependent manner. Actinomycin D, cyclohexamide and retinoic acid had inhibitory effects on MMP-2, while dexamethasone showed slight stimulatory effect on MMP-2 secretion. Our results showed that select cytokines, mitogens and inhibitors modulated A-2058 MMP-2 and MMP-9 expression. They suggest the clinical potential of MMP inhibitors, especially the non-toxic ones, such as the nutrient mixture and its component EGCG in management of melanoma.

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Introduction

Cancer of the skin is by far the most common of all cancers. Melanoma accounts for only ~1% of skin cancers but causes a large majority of skin cancer deaths, due to metastasis to other areas of the body, such as lymph nodes, lungs, liver, brain or bone. Though often curable in its early stages, metastatic malignant melanoma is an extremely aggressive cancer with no current viable treatment. The American Cancer Society estimates that 76,380 new melanomas will be diagnosed (~46,870 in men and 29,510 in women) in the United States for 2016. Approximately 10,130 people are expected to die of melanoma (~6,750 men and 3,380 women). The rates of melanoma have been rising for the last 30 years (1).

Thus, any successful treatment for melanoma has to target metastasis, which is dependent upon degradation of the extracellular matrix (ECM), which, when intact, acts as a barrier to block cancer cell invasion (2-4). Clinical and experimental studies have demonstrated that elevated levels of matrix metalloproteinases are associated with rapid progression of metastatic melanoma (5,6). MMP activity is regulated by and dependent upon environmental influences from surrounding stroma cells, ECM proteins, systemic hormones and other factors. Inflammation has been reported to drive cancer progression (7-9). Inflammatory cytokines such as interleukin (IL)-1β play significant roles in inflammation driven melanoma growth and progression (10). In the present study we investigated the effects of selected cytokines, inducers and inhibitors affecting cancer cell metabolism on the regulation of MMP-2 and MMP-9 activities in melanoma A-2058 cell line.

Materials and methods

Materials. Human melanoma A-2058 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Antibiotics, penicillin and fetal bovine serum (FBS), were obtained from Gibco-BRL (Long Island, NY, USA). Twenty-four well tissue culture plates were obtained from Corning Costar Corp. (Cambrdige, MA, USA). Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen) with 0.1% gelatin in non-reducing conditions. Interleukin 1β, tumor necrosis factor-α (TNF-α), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), doxycycline, epigallocatechin gallate (EGCG), actinomycin-D, cyclohexamide, retinoic acid and dexamethasone, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The nutrient mixture (NM), prepared by VitaTech (Hayward, CA, USA) 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 1,000 mg; L-proline, 750 mg; L-arginine, 500 mg; N-acetyl cysteine, 200 mg; standardized green tea extract (80% polyphenol), 1,000 mg; selenium, 30 μg; copper, 2 mg; manganese, 1 mg. All other reagents used were of high quality and were obtained from Sigma-Aldrich, unless otherwise indicated.

Cell cultures. Melanoma cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in 24-well tissue culture plates. The cells were plated at a density of $1x10^5$ cells/ml and grown to confluency in a humidified atmosphere at 5% CO_2 at 37˚C. Serum-supplemented media were removed and the cell monolayer was washed once with phosphate-buffered saline (PBS) and with the recommended serum-free media. The cells were then incubated in 0.5 ml of serum-free medium with various cytokines, mitogens, inducers and inhibitors in triplicate, as indicated: PMA (10, 25, 50 and 100 ng/ml); TNF-α and IL-1β (0.1, 1, 10 and 25 ng/ml); LPS (10, 25, 50 and 100 μg/ml); EGCG (10, 25, 50 and 100 μM) without and with PMA 100 ng/ml; doxycycline (10, 25, 50 and 100 μM) without and with PMA 100 ng/ml; NM (10, 50, 100, 500 and 1,000 μg/ml) without and with PMA 100 ng/ml, retinoic acid (50 μM); dexamethasone (50 μM); actinomycin D and cyclohexamide (2 and 4 μg/ml). The plates were then returned 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, Inc., (Orem, UT, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi).

Statistical analysis. Microsoft Excel 2010 linear trend analysis was utilized to determine the linear trend analyses of the densitometry results.

Table I. Effect of inducers, cytokines and mitogens on melanoma A-2058 MMP-2 and MMP-9 secretion.

Treatment	MMP-2 (%)	MMP-9 (%)
PMA (ng/ml)		
Control	100	100
10	92.2	100.9
25	89.7	146.8
50	94.6	288.5
100	97.6	399.9
TNF-α (ng/ml)		
Control	100	100
0.1	99.7	118.4
1	99.7	118.4
10	98.9	145.1
25	97.8	204.2
IL-1β (ng/ml)		
Control	100	100
0.1	118.3	99.8
1	117.4	99.6
10	122.1	99.7
25	64.6	51.2
LPS (μg/ml)		
Control	100	100
10	93.3	172
25	103	228
50	106	200
100	103	161

Results

Inducers, mitogens and cytokines. Melanoma A-2058 expressed bands corresponding to MMP-2 and MMP-9. Table I shows the quantitative densitometry results from the effects of

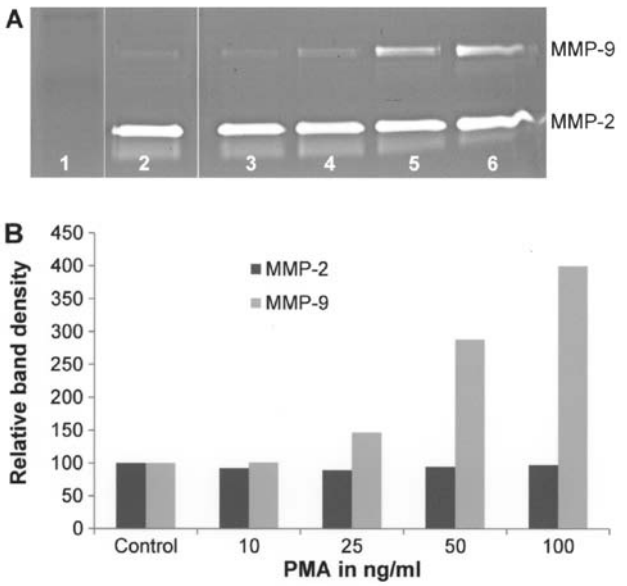

Figure 1. Effect of PMA on MMP-2 and -9 secretions in melanoma A-2058 cell line. (A) Gelatinase zymogram and (B) densitometry analysis of A-2058 MMP-2 and -9 expressions. Lane 1, markers; lane 2, Control; lanes 3-6 PMA (10, 25, 50 and 100 ng/ml, respectively).

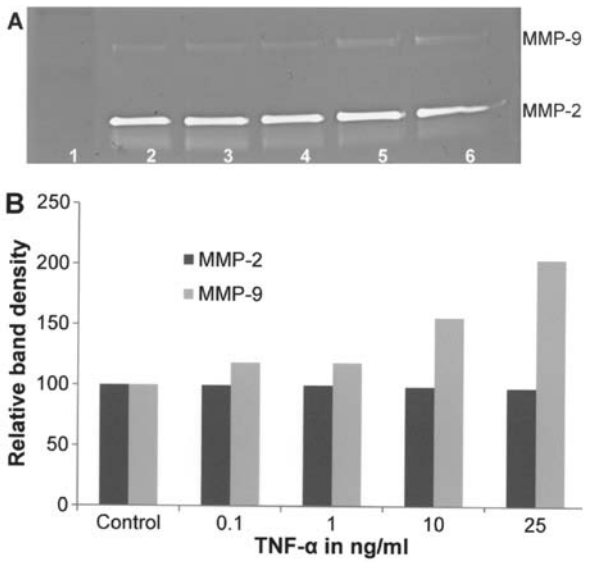

Figure 2. Effect of TNF-α on MMP-2 and -9 secretions in melanoma A-2058 cell line. (A) Gelatinase zymogram and (B) densitometry analysis of A-2058 MMP-2 and MMP-9 expressions. Lane 1, markers; lane 2, Control; lanes 3-6, TNF-α (0.1, 1, 10 and 25 ng/ml, respectively).

PMA, TNF-α, IL-1β and LPS on MMP-2 and MMP-9 expression in A-2058 cells.

Effect of PMA on A-2058 cell secretion of MMPs. On gelatinase zymography, A-2058 demonstrated strong expression of MMP-2 and slight expression of MMP-9. PMA treatment ranging from concentrations of 10-100 ng/ml showed no significant effect on MMP-2 secretion (linear trend R^2=0.009) but significant dose-dependent upregulation of MMP-9 secretion with 400% that of control at 100 ng/ml (linear trend R^2=0.883) (Fig. 1).

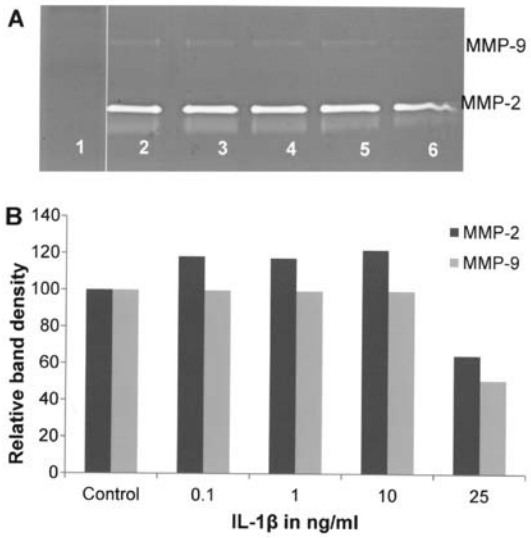

Figure 3. Effect of IL-1β on MMP-2 and -9 secretions in melanoma A-2058 cell line. (A) Gelatinase zymogram and (B) densitometry analysis of A-2058 MMP-2 and MMP-9 expressions. Lane 1, Markers; lane 2, Control; lanes 3-6, IL-1β (0.1, 1, 10 and 25 ng/ml, respectively).

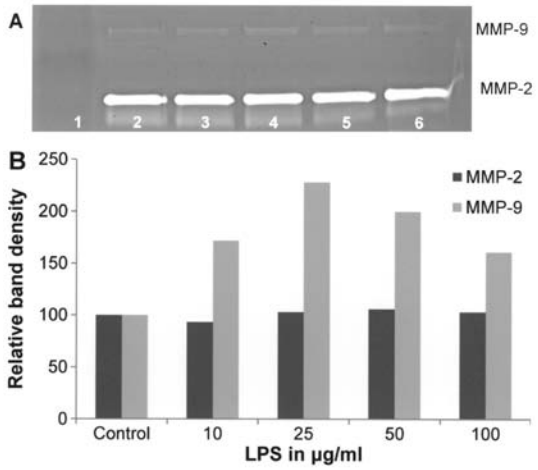

Figure 4. Effect of LPS on MMP-2 and -9 secretions in melanoma A-2058 cell line. (A) Gelatinase zymogram and (B) densitometry analysis of A-2058 MMP-2. Lane 1, markers; lane 2, Control; lanes 3-6, IL-1β (0.1, 1, 10 and 25 ng/ml, respectively).

Effect of TNF-α on A-2058 cell secretion of MMPs. On gelatinase zymography, A-2058 demonstrated strong expression of MMP-2 and slight expression of MMP-9. TNF-α, used between 0.1-25 ng/ml, showed no significant overall effect on expression of MMP-2 (linear trend R^2=0.037). TNF-α showed dose-dependent increased MMP-9 secretion (linear trend R^2=0.876) with 200% of control at 25 ng/ml (Fig. 2).

Effect of IL-1β on A-2058 cell secretion of MMPs. Gelatinase zymography demonstrated strong expression of MMP-2 by A-2058 cells and slight expression of MMP-9. IL-1β at a concentration range of 0.1-25 ng/ml did not have significant effect on MMP-2 secretion by A-2058 cells, except at 25 ng/ml where MMP-2 level was reduced by ~40% (linear trend R^2=0.529). Also, MMP-9 secretion was not modified by IL-1β except for its ~50% reduction at 25 ng/ml (Fig. 3).

Table II. Effect of inhibitors on melanoma A-2058 MMP-2 and MMP-9 secretion.

	Untreated		PMA 100 ng/ml-treated	
Treatment	MMP-2 (%)	MMP-9 (%)	MMP-2 (%)	MMP-9 (%)
Doxycycline (μM)				
Control	100	100	100	100
10	89.8	99.3	102	122.6
25	89.2	105.5	95	111.2
50	66.5	97.9	69.8	68.7
100	28.3	70.1	33.2	29.5
EGCG (μM)				
Control	100	100	100	100
10	98.8	97.8	95.3	87.8
25	101.9	104.4	88.6	70.5
50	81.3	95.5	56.2	43.5
100	42.6	26.8	35	1
NM (μg/ml)				
Control	100		100	100%
10	112.8		119.9	87.7
50	92.4		82.8	17.4
100	26.6		42.3	14.4
500	12.3		13.1	2
1,000	0		4.1	1
Actinomycin D (μg/ml)				
Control	100			
2	74.3			
4	72.6			
Cyclohexamide (μg/ml)				
Control	100			
2	17.4			
4	19.9			
Dexamethasone (μM)				
Control	100			
50	107			
Retinoic acid (μM)				
Control	100			
50	0			

Effect of LPS on A-2058 cell secretion of MMPs. Gelatinase zymography demonstrated strong expression of MMP-2 and slight expression of MMP-9 by A-2058 melanoma cells. LPS treatment between 10-100 μg/ml showed no significant effect on MMP-2 secretion (linear trend R^2=0.375). However, enhanced MMP-9 secretion was observed at LPS up to 25 μg/ml that was followed by a decreased MMP-9 level (linear trend R^2=0.244) (Fig. 4).

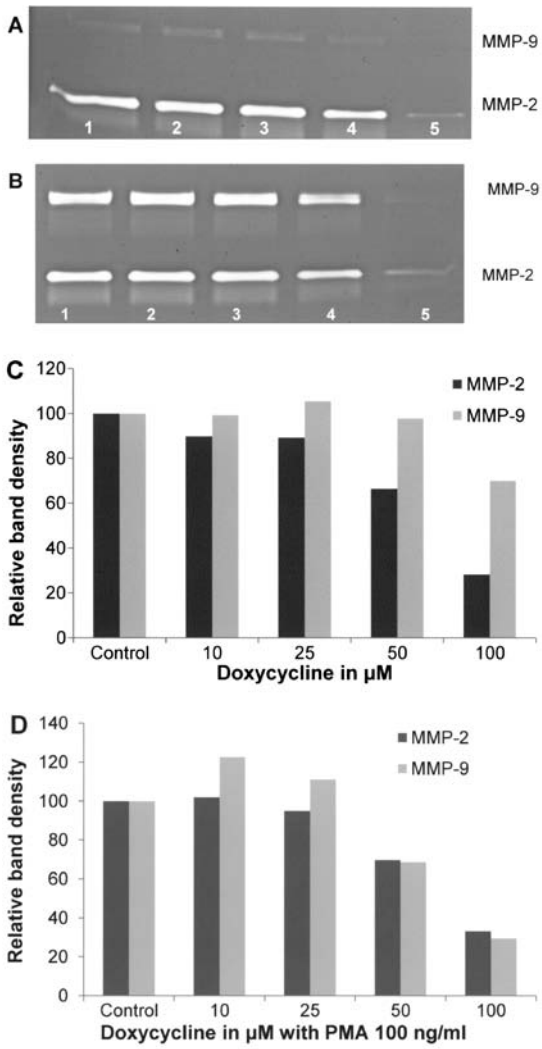

Figure 5. Effect of doxycycline on MMP-2 and -9 secretion by normal and PMA 100 ng/ml-treated cells in melanoma A-2058 cell line. (A) Gelatinase zymograms of normal A-2058 cells and (B) PMA-treated A-2058 cells and densitometry analyses (C) of normal A-2058 cells and (D) PMA-treated A-2058 cells. Lane 1, Control; lanes 2-5, doxycycline (10, 25, 50 and 100 μM, respectively).

Chemical inhibitors. Table II shows the quantitative densitometry results from the effects of select chemical inhibitors doxycycline, dexamethasone, actinomycin D and cyclohexamide on MMP-2 and MMP-9 expression in melanoma A-2058 cell line.

Effect of doxycycline on A-2058 cell secretion of MMPs. Doxycycline inhibited MMP-2 secretion by A-2058 cells in a dose-dependent manner with 72% blockage at 100 μM (linear trend R^2=0.843). MMP-9 secretion was not affected by doxycycline up to 50 μM, and at 100 μM it was decreased by 30% (linear trend R^2=0.480). In the presence of PMA at 100 ng/ml, doxycycline downregulated the expression of MMP-2 in a dose-dependent manner, with 67% block (R^2=0.8072) at 100 μM and 70% block (linear trend R^2=0.6711) of MMP-9 at 100 μM (Fig. 5).

Effect of actinomycin D on A-2058 cell secretion of MMPs. Actinomycin D had moderate inhibitory effect on A-2058

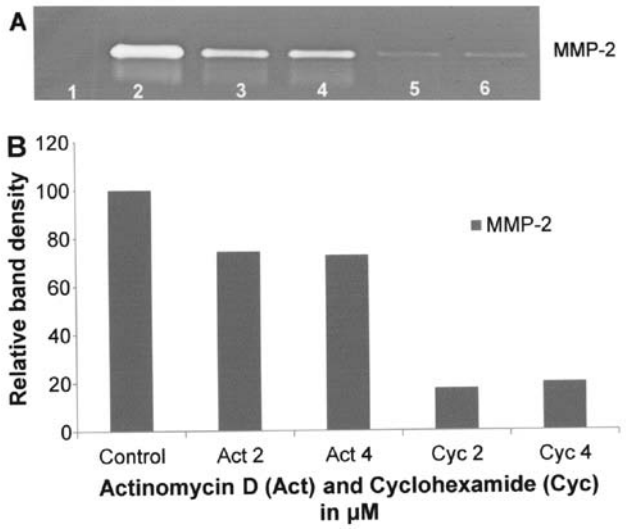

Figure 6. Effect of actinomycin D and cyclohexamide on MMP-2 secretion by normal cells in melanoma A-2058 cell line. (A) Gelatinase zymogram of normal A-2058 cells and (B) densitometry analysis. Lane 1, markers; lane 2, control; lanes 3 and 4, actinomycin D (2 and 4 μM, respectively); lanes 5 and 6, cyclohexamide (2 and 4 μM, respectively).

MMP-2 secretion (R^2=0.797) with 26 and 27% inhibition at 2 and 4 μM, respectively, as shown in Fig. 6.

Effect of cyclohexamide on A-2058 cell secretion of MMPs. Cyclohexamide had potent dose-dependent inhibitory effect on MMP-2 secretion, resulting in its inhibition by 80% at 4 μM (linear trend R2=0.727), as shown in Fig. 6.

Effect of dexamethasone on A-2058 cell secretion of MMPs. Dexamethasone had slight stimulatory effect on MMP-2, with 107% of control at 50 μM (data not shown).

Natural inhibitors. Table II shows the quantitative densitometry results, presenting the effects of natural compounds EGCG alone, the EGCG in a complex with other natural compounds (NM) and retinoic acid on MMP-2 and MMP-9 expression in melanoma A-2058 cells.

Effect of EGCG on A-2058 cell secretion of MMPs. Fig. 7 shows the effects of EGCG at 10, 25, 50 and 100 μM concentrations on MMP-2 and MMP-9 secretions by unstimulated and PMA-stimulated A2058 melanoma cells. EGCG showed inhibitory effects on MMP-2 and -9 secretions by melanoma cells in a dose-dependent manner. In the presence of 100 μM EGCG, the secretion of MMP-2 decreased by 57.4% (linear trend R^2=0.697) and MMP-9 by 73.2% (linear trend R^2=0.519), as shown in Fig. 7A and C. In the presence of PMA (100 ng/ml) the secretion of these enzymes was inhibited by EGCG in a dose-dependent manner. Slight inhibitory effect of EGCG on PMA-induced MMP-9 secretion was already observed at 25 μM (30% inhibition) and its total block at 100 μM (linear trend R^2=0.901), as shown in Fig. 7B and D.

Effect of NM on A-2058 cell secretion of MMPs. As shown in Fig. 8A and C, NM was effective in inhibiting secretion of MMP-2 by uninduced A-2058 cells in a dose-dependent

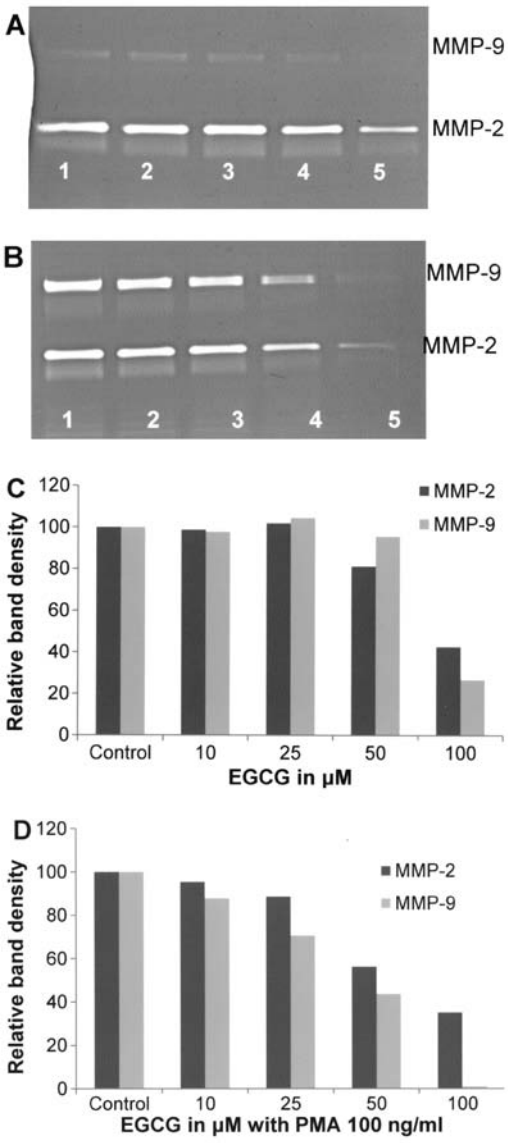

Figure 7. Effect of EGCG on MMP-2 and -9 secretion by normal and PMA 100 ng/ml-treated cells in melanoma A-2058 cell line. (A) Gelatinase zymograms of normal A-2058 cells and (B) PMA-treated A-2058 cells and densitometry analyses (C) of normal A-2058 cells and (D) PMA-treated A-2058 cells. Lane 1, Control; lanes 2-5, EGCG (10, 25, 50 and 100 μM, respectively).

manner when used at 10, 50, 100, 500 and 1,000 μg/ml (linear trend R^2=0.868) with virtual total block at 1,000 μg/ml. NM also showed dose-dependent inhibition of MMP-2 and -9 expression in PMA-treated A-2058 cells as presented in Fig. 8B and D, which showed 96% blockage of MMP-2 secretion at 1,000 μg/ml and total block of MMP-9 at 1,000 μg/ml (linear trends R^2=0.889 and 0.818, respectively).

Effect of retinoic acid on A-2058 cell secretion of MMPs. Retinoic acid inhibited A-2058 MMP-2 secretion with total block at 50 μM (data not shown).

Discussion

Elevated MMP levels correlate with melanoma tumor progression, as documented in clinical studies (5,6). Thus, knowledge

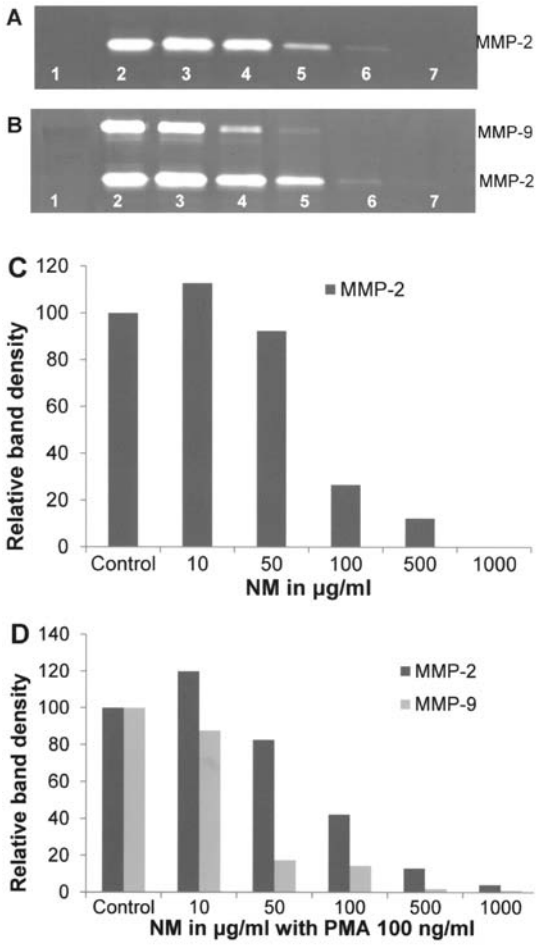

Figure 8. Effect of NM on MMP-2 and -9 secretion by normal and PMA 100 ng/ml-treated cells in melanoma A-2058 cell line. (A) Gelatinase zymograms of normal A-2058 cells and (B) PMA-treated A-2058 cells and densitometry analyses (C) of normal A-2058 cells and (D) PMA-treated A-2058 cells. Lane 1, markers; lane 2, Control; lanes 3-7, NM (10, 50, 100, 500 and 1,000 μg/ml, respectively).

of MMP regulation is of importance for developing therapeutic strategies for melanoma. Nikkola *et al* (5) found that melanoma patients with high-serum levels of MMP-9 had significantly poorer overall survival than patients with lower serum MMP-9 levels and that high MMP-9 levels were correlated with visceral or bone metastasis and presence of liver metastases. Malaponte *et al* (6) found that plasma levels of MMP-2 and TGF-β in patients with primary melanoma were significantly higher than those of healthy controls and those significantly higher levels were found in patients with metastatic melanoma. Extracellular factors, such as the inflammatory cytokine IL-1β has been implicated in facilitating inflammation and melanoma tumor growth (10).

In the present study, we compared MMP secretion patterns in the presence of cytokines, PMA and LPS in melanoma A-2058 cells. In addition, we investigated the effect of inhibitors doxycycline, EGCG, NM and other compounds, such as dexamethasone, cyclohexamide, retinoic acid and agents that affect protein transcription and translation levels, such as actinomycin D. None of the tested inducers and cytokines was found to enhance MMP-2 secretion. PMA and TNF-α treatment showed potent dose-dependent upregulation of MMP-9 secretion up to 400% that of control at 100 ng/ml PMA and 200% of control at 25 ng/ml TNF-α. Surprisingly, IL-1β demonstrated no effect on MMP-9 secretion, except its decrease at 25 ng/ml and LPS showed no consistent effect, resulting in an upregulation and downregulation of MMP-9 secretion. Among tested inhibitors, chemical inhibitor doxycycline and natural inhibitors EGCG and NM with and without PMA treatment, potently downregulated the secretion of A-2058 MMP-2 and MMP-9 in a dose-dependent manner. MMP-2 secretion was potently inhibited by cyclohexamide and retinoic acid, moderately inhibited by actinomycin D and slightly stimulated by dexamethasome.

In addition to individual compounds we tested the effects of a specific nutrient mixture (NM) which has demonstrated anticancer efficacy in various *in vitro* and *in vivo* studies by affecting various mechanisms, including MMPs secretion (11). EGCG from green tea is one of its components, with green tea extract comprising ~23% total NM weight. Another component is amino acid lysine, which is a natural inhibitor of plasmin-induced proteolysis, including MMPs (12,13). Lysine also contributes to 23% of weight in NM. These and other compounds in NM (i.e. vitamin C, lysine, proline, copper, manganese and N-acetyl cysteine) are important in supporting the integrity and stability of connective tissue through various mechanisms, thereby contributing to tumor encapsulation and curtailing cancer cells spread (14-22). In addition, NM components N-acetyl cysteine and selenium have been shown to inhibit tumor cell MMP-9 and invasive activities and migration of endothelial cells through the ECM (23-25). Ascorbic acid has been documented to modulate cancer cell and tumor growth as well as to prevent metastasis (26-31) and low levels of ascorbic acid are found in cancer patients (32-34). The inhibitory effects of NM in comparison to EGCG alone on MMP-9 and MMP-2 secretion in unstimulated and PMA-stimulated A-2058 cells suggest enhancing effectiveness of EGCG when applied in a mixture with other natural compounds of similar and complementary mechanisms. The contribution of individual NM components towards inhibition of MMPs warrants further investigation.

In conclusion, our results showed that various inducers, cytokines, mitogens and inhibitors tested in this study modulate MMP-2 and -9 secretions by melanoma A-2058 cells. They suggest the clinical use of MMP inhibitors, especially potent and non-toxic ones as the nutrient mixture (NM) and its component EGCG in management of melanomas.

Acknowledgements

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