Cytokines, inducers and inhibitors modulate MMP-2 and MMP-9 secretion by human Fanconi anemia immortalized fibroblasts

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Abstract. Acute myeloid leukemia and head and neck squamous cell carcinomas are the major causes of mortality and morbidity in Fanconi anemia (FA) patients. Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, have been implicated in tumor invasion and metastasis. Various cytokines, mitogens, growth factors, inducers and inhibitors control MMP activities. We investigated the roles of these in the regulation of MMP-2 and MMP-9 in human immortalized fibroblasts from FA. Human FA immortalized fibroblast cell lines FA-A:PD220 and FA-D2:PD20 were grown in minimum essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and antibiotics in 24-well tissue culture plates. At near confluence, the cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free media with the following: phorbol 12-myristate 13-acetate (PMA) at 10-100 ng/ml; tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) at 0.1-25 ng/ml; lipopolysaccharide (LPS) at 10-100 μg/ml; epigallocatechin gallate (EGCG) and doxycycline (Dox) at 10-100 μM without and with PMA; a nutrient mixture (NM) without and with PMA at 10-1,000 μg/ml; actinomycin-D and cyclohexamide at 2 and 4 μM; retinoic acid and dexamethasone at 50 μM. After 24 h, media were removed and analyzed for MMP-2 and MMP-9 by zymography. Both FA cell lines expressed only MMP-2 and responded similarly to cytokines, mitogens, inducers and inhibitors. PMA potently stimulated MMP-9 and had a moderate effect on MMP-2. TNF-α showed variable effects on MMP-2 and significantly enhanced MMP-9. IL-1β enhanced MMP-2 slightly and MMP-9 significantly. LPS had a moderate stimulatory effect on MMP-2 and no effect on MMP-9. EGCG, Dox and NM, without and with PMA, downregulated MMP-2 and MMP-9 expression. Actinomycin-D, retinoic acid and dexamethasone also had inhibitory effects on MMP-2. Our results showed that cytokines, mitogens and inhibitors modulated FA fibroblast MMP-2 and MMP-9 expression, suggesting the clinical use of MMP inhibitors, particularly such potent and non-toxic ones as the NM and its component EGCG in the management of FA cancers.

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Introduction

Fanconi anemia (FA) is a heterogeneous autosomal recessive disease characterized by congenital malformations, progressive bone marrow failure and an increased incidence of cancer. In contrast to normal fibroblasts, FA fibroblasts display elevated spontaneous chromosomal breaks and deletions and nuclear extracts that have substantially decreased plasmid-rejoining activity (1,2). Epanchintsev *et al* demonstrated the overproduction of secretory factors such as interleukin (IL)-6, IL-8, matrix metalloproteinase (MMP)-2, and MMP-9 in FA and showed that these overexpressed secretory factors were effective in promoting the proliferation, migration and invasion of surrounding tumor cells (3). Ibáñez *et al* described an anomalous high level of the pro-inflammatory cytokine IL-1β present in the serum of FA patients which activated the proliferation of tumor cells (4). Increased levels of MMP-9 have been shown to be associated with cancer progression and poorer patient prognosis due to the significant role MMP-9 plays in tumor cell invasion and metastasis by digesting the basement membrane and components of the extracellular matrix (5-7). MMP activity is regulated by and dependent upon environmental influences from surrounding stroma cells, ECM proteins, systemic hormones and other factors (5,8,9). Furthermore, MMPs are regulated at multiple levels, including transcription, modulation of messenger RNA half-life (translation), secretion, localization, activation and inhibition (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 FA fibroblast cell lines.

Materials and methods

Materials. Human FA fibroblast cell lines A:PD20 and A:PD220 were obtained from the Fanconi Anemia Research Fund, Oregon Health & Science University (Portland, OR, 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

Costar (Cambridge, MA, USA). Gelatinase zymography was performed on 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen Inc., Carlsbad, CA, USA) with 0.1% gelatin in non-reducing conditions. IL-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. FA fibroblasts 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 of 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 triplicates, as indicated: PMA (10, 25, 50 and 100 ng/ml); TNF-α (0.1, 1, 10 and 25 ng/ml); 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) 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 separately collected, 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 due to 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 on 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 a 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 (Orem, UT, USA), at a resolution of 1 scanner unit (1/100 of an inch for an image that was scanned at 100 dpi).

Table I. Effect of inducers on Fanconi anemia fibroblast MMP-2 and MMP-9 secretion.

Inducers	MMP-2 (%)	MMP-9 (%)
PMA (ng/ml)		
Control	100	0
10	126	16.5
25	217	33.5
50	236	32
100	167	19
TNF-α (ng/ml)		
Control	100	100
0.1	156	365
1	140	622
10	90	5,660
25	92	6,487
IL-1β (ng/ml)		
Control	100	100
0.1	82	145
1	121	880
10	173	1,216
25	111	700
LPS (μg/ml)		
Control	100	
10	118	
25	170	
50	104	
100	65	

MMPs, matrix metalloproteinases; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; LPS, lipopolysaccharide.

Results

Inducers and cytokines. Both FA cell lines A:PD20 and A:PD220 expressed only one band corresponding to MMP-2. Cytokines, mitogens, inducers and inhibitors had a similar effect on MMP-2 and PMA-induced MMP-9 expression in both FA fibroblasts. Therefore, only data for FA A:PD20 is presented. Table I shows the quantitative densitometry results from the effects of PMA, TNF-α, IL-1β and LPS on MMP-2 and MMP-9 expression in the FA fibroblasts.

Effect of PMA on FA fibroblast secretion of MMPs. Upon gelatinase zymography, FA fibroblasts demonstrated moderate

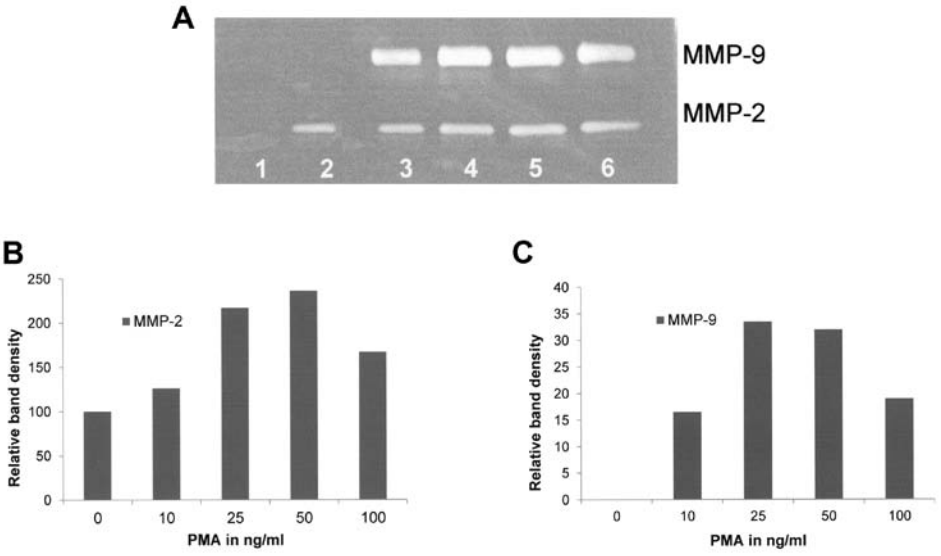

Figure 1. Effect of PMA on MMP-2 and MMP-9 expression in the FA-A:PD20 cell line. (A) Gelatinase zymogram of MMP-2 and MMP-9. Densitometry analysis of FA fibroblast (B) MMP-2 and (C) MMP-9 expression levels. Lane 1, markers; lane 2, control; lanes 3-6: 10, 25, 50, 100 ng/ml of PMA, respectively. PMA, phorbol 12-myristate 13-acetate; MMP, matrix metalloproteinase; FA, Fanconi anemia.

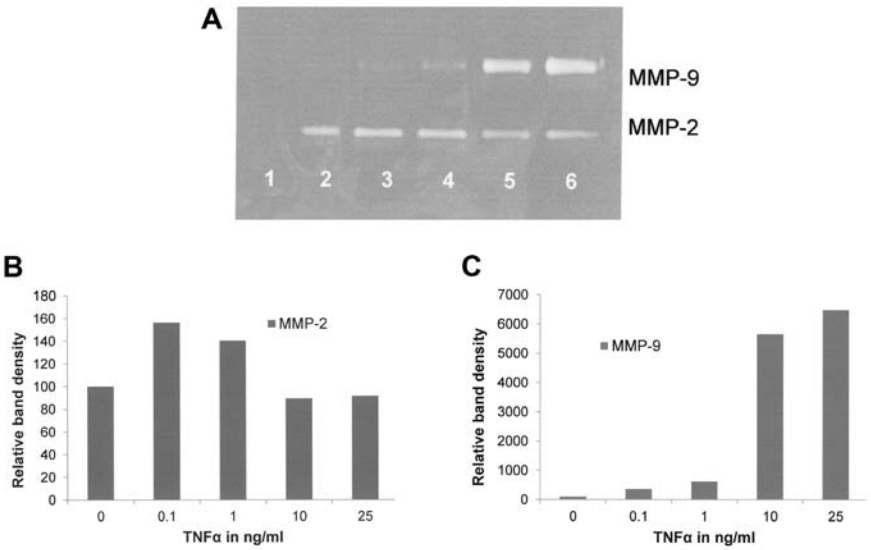

Figure 2. Effect of TNFα on MMP-2 and MMP-9 secretion in the FA-A:PD20 cell line. (A) Gelatinase zymogram of MMP-2 and MMP-9 secretion. Densitometry analysis of FA fibroblast (B) MMP-2 and (C) MMP-9 expression levels. Lane 1, markers; lane 2, control; lanes 3-6: 0.1, 1, 10 and 25 ng/ml of TNFα, respectively. TNF-α, tumor necrosis factor-α; MMP, matrix metalloproteinase; FA, Fanconi anemia.

expression of MMP-2 and no expression of MMP-9. PMA treatment had a moderate stimulatory effect on the expression of MMP-2 (linear trend R^2=0.4446) and strongly stimulated MMP-9 expression in a dose-dependent manner (linear trend R^2=0.4084) as shown in Fig. 1.

Effect of TNFα on FA fibroblast secretion of MMPs. TNF-α had a negligible effect on MMP-2 (R^2=0.1844) and a significant stimulatory dose-dependent effect on MMP-9 (R^2=0.824) as shown in Fig. 2.

Effect of IL-1β on FA fibroblast secretion of MMPs. IL-1β caused slight stimulation of MMP-2 at 1 and 10 ng/ml (R^2=0.273), and had significant stimulatory dose-dependent effects on MMP-9 (R^2=0.558) as shown in Fig. 3.

Effect of LPS on FA fibroblast secretion of MMPs. LPS had a moderate stimulatory effect on MMP-2 secretion below 50 μg/ml and an inhibitory effect at 50 and 100 μg/ml (Fig. 4) and no effect on MMP-9 (data not shown).

Chemical inhibitors. Table II shows the quantitative densitometry results from the effects of the chemical inhibitors doxycycline, dexamethasone and actinomycin-D on MMP-2 and MMP-9 expression in FA fibroblast cell lines.

Doxycycline showed increased FA fibroblast MMP-2 secretion at 10 and 25 μM, and decreased secretion at 50 and 100 μM with virtual total blockage at 100 μM (R^2=0.296). When treated with PMA 100 ng/ml, doxycycline downregulated the expression of FA fibroblast MMP-2 and MMP-9 in a dose-dependent manner, with virtual total blockage of

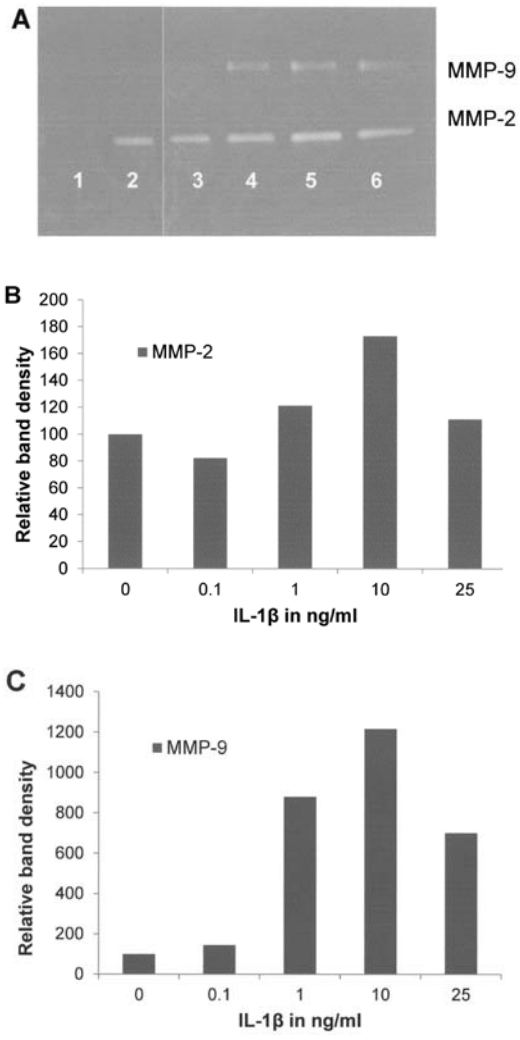

Figure 3. Effect of IL-1β on MMP-2 and MMP-9 secretion in the FA-A:PD20 cell line. (A) Gelatinase zymogram of MMP-2 and MMP-9 secretion. Densitometry analysis of FA fibroblast (B) MMP-2 and (C) MMP-9 expression levels. Lane 1, markers; lane 2, control; lanes 3-6: 0.1, 1, 10 and 25 ng/ml of IL-1β, respectively. IL-1β, interleukin-1β; MMP, matrix metalloproteinase; FA, Fanconi anemia.

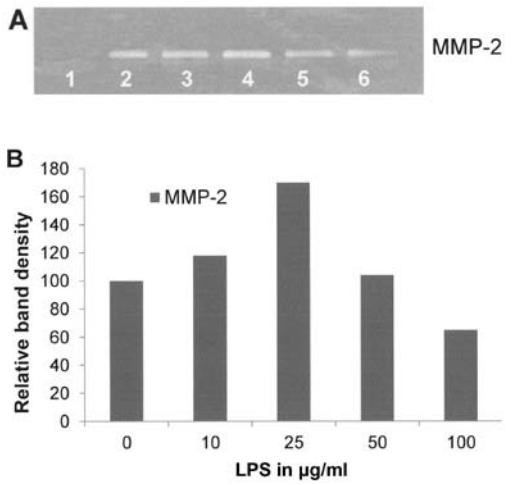

Figure 4. Effect of LPS on MMP-2 secretion in the FA-A:PD20 cell line. (A) Gelatinase zymogram and (B) densitometry analysis of FA fibroblast MMP-2. Lane 1, markers; lane 2, control; lanes 3-6: 10, 25, 50 and 100 µg/ml of LPS, respectively. LPS, lipopolysaccharide; MMP, matrix metalloproteinase; FA, Fanconi anemia.

Table II. Effect of inhibitors on Fanconi anemia fibroblast MMP-2 and MMP-9 secretion.

	Untreated	PMA-treated (100 ng/ml)	
Inhibitors	MMP-2 (%)	MMP-2 (%)	MMP-9 (%)
Doxycycline (μM)			
Control	100	100	100
10	265	59	4
25	255	55	7
50	49	8	0.5
100	1	0.5	0.5
EGCG (μM)			
Control	100	100	100
10	201	118	16
25	148	107	13
50	75	39	1
100	17	0.5	0.5
NM (μg/ml)			
Controls	100	100	100
10	112	98	53
50	80	102	30
100	19	49	10
500	1	1	1
1,000	1	1	1
Dexamethasone (μM)			
Control	100		
50	12		
Retinoic acid (μM)			
Control	100		
50	7		
Actinomycin-D (μM)			
Control	100		
2	56		
4	67		

MMPs, matrix metalloproteinases; EGCG, epigallocatechin gallate; NM, nutrient mixture.

MMP-2 at 100 μM (R^2=0.9378) and of MMP-9 at 50 μM (R^2=0.5403) as shown in Fig. 5. Actinomycin-D had a slight inhibitory effect on MMP-2 (R^2=0.5355) with 33% inhibition at 4 μM as shown in Fig. 6. Dexamethasone had a potent inhibitory effect on MMP-2, with inhibition of 88% at 50 μM compared to the control (data not shown). Cyclohexamide had no effect on MMP-2 secretion by FA fibroblasts (data not shown).

Natural inhibitors. Table II shows the quantitative densitometry results from the effects of natural inhibitors EGCG, NM and retinoic acid on MMP-2 and MMP-9 expression in FA fibroblast cell lines.

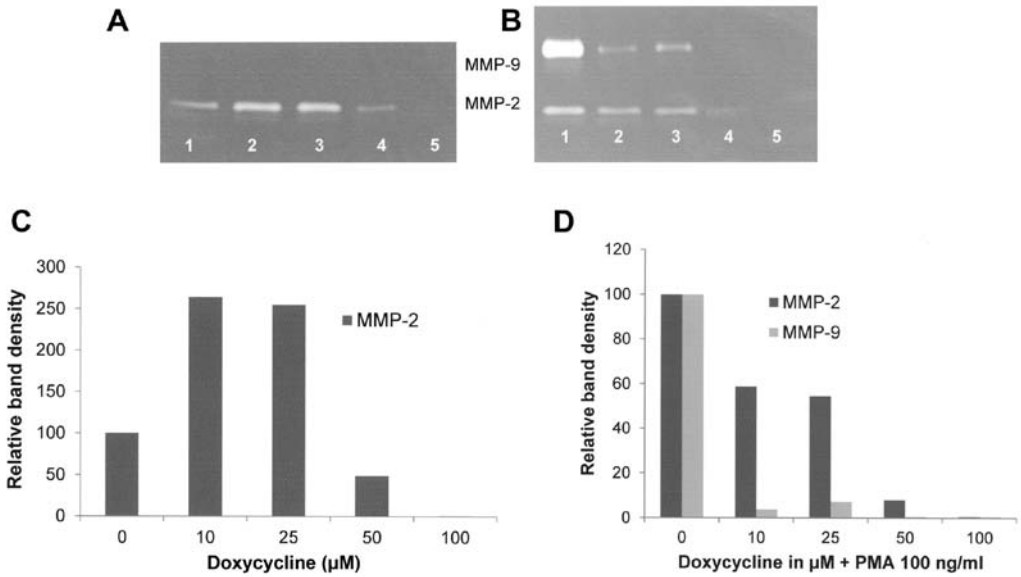

Figure 5. Effect of doxycycline on MMP-2 and MMP-9 secretion of normal and PMA-treated (100 ng/ml) FA-A:PD20 cells. (A) Gelatinase zymograms of normal FA fibroblast cells. (B) PMA-treated FA fibroblast cells. (C) Densitometry analysis of normal FA fibroblast cells and (D) PMA-treated FA fibroblast cells. Lane 1, markers; lane 2, control; lanes 2-5, 10, 25, 50 and 100 μM of doxycycline, respectively. PMA, phorbol 12-myristate 13-acetate; MMP, matrix metalloproteinase; FA, Fanconi anemia.

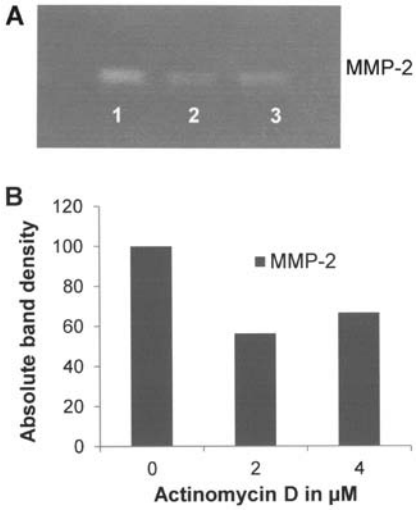

Figure 6. Effect of actinomycin-D on MMP-2 and MMP-9 secretion of normal FA-A:PD20 cells. (A) Gelatinase zymograms of normal FA fibroblast cells and (B) densitometry analysis of MMP-2. Lane 1, control; lanes 2 and 3: 2 and 4 μM of actinomycin-D, respectively. MMP, matrix metalloproteinase; FA, Fanconi anemia.

EGCG downregulated the expression of MMP-2 at and over 50 μM, with 83% block at 100 μM (R^2=0.4337) as shown in Fig. 7A and C. EGCG showed inhibition of PMA-induced (100 ng/ml) MMP-9 (R^2=0.6554) and of MMP-2 (R^2=0.7476) in a dose-dependent manner with virtual total block of both at 100 μM as shown in Fig. 7B and D.

NM inhibited the secretion of MMP-2 by uninduced FA fibroblast cells in a dose-dependent manner, with a linear trend of R^2=0.8706 (Fig. 8A and C). NM showed dose-dependent inhibition of MMP-2 and MMP-9 expression in PMA-treated cells with virtual total blockage of both at 500 μg/ml as shown in Fig. 8B and C, with linear trends R^2=0.8479 and 0.8597, respectively.

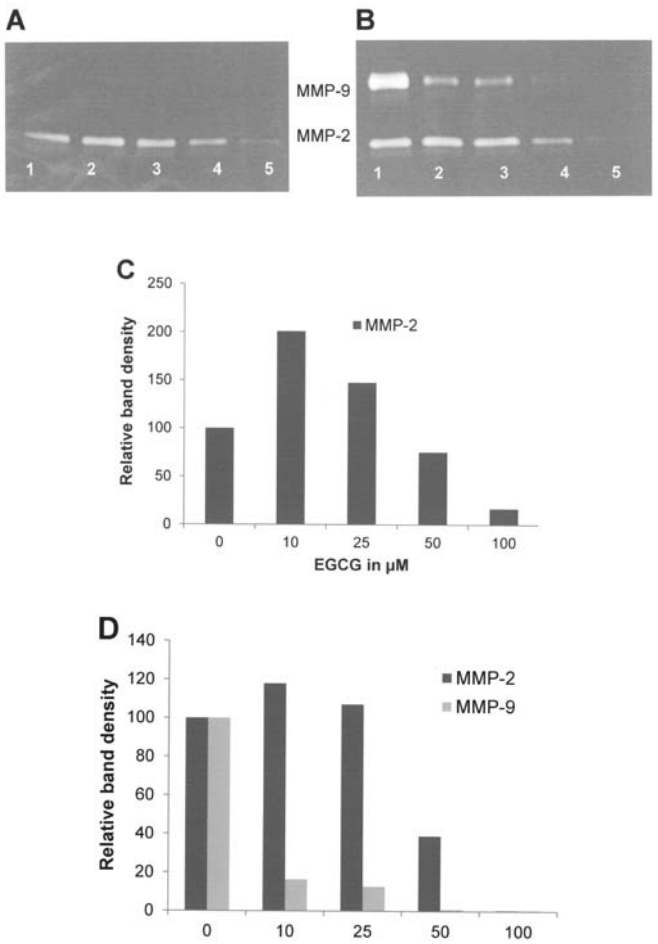

Figure 7. Effect of EGCG on MMP-2 and MMP-9 secretion of normal and PMA-treated (100 ng/ml) FA-A:PD20 cells. (A) Gelatinase zymograms of normal FA fibroblast cells and (B) PMA-treated FA fibroblast cells. (C) Densitometry analysis of normal FA fibroblast cells and (D) PMA-treated FA fibroblast cells. Lane 1, markers; lane 2, control; lanes 2-5: 10, 25, 50 and 100 μM of EGCG, respectively. EGCG, epigallocatechin gallate; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; FA, Fanconi anemia.

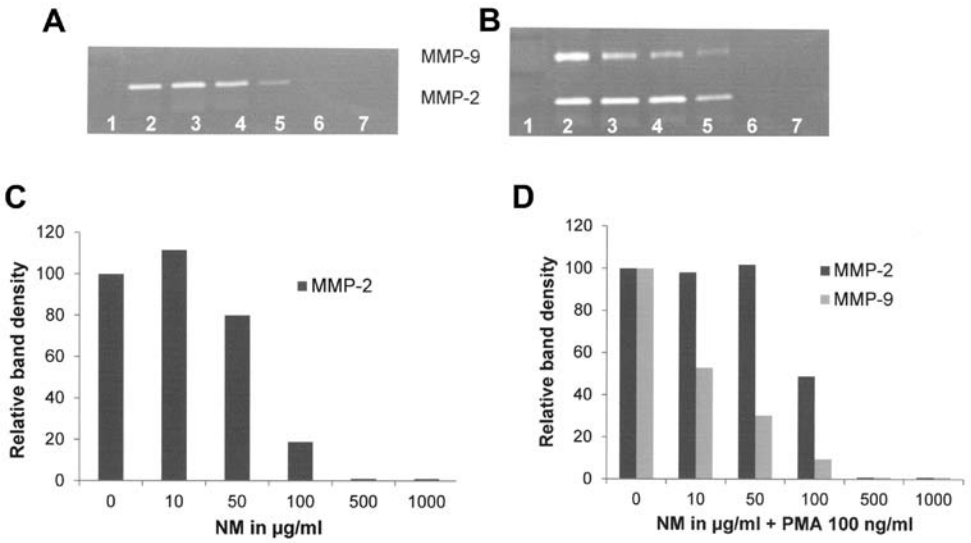

Figure 8. Effect of NM on MMP-2 and MMP-9 secretion of normal and PMA-treated (100 ng/ml) FA-A:PD20 cells. (A) Gelatinase zymograms of normal FA fibroblast cells and (B) PMA-treated FA fibroblast cells. (C) Densitometry analysis of normal FA fibroblast cells and (D) PMA-treated FA fibroblast cells. Lane 1, markers; lane 2, control; lanes 3-7: 10, 50, 100, 500 and 1,000 μg/ml of NM, respectively. NM, nutrient mixture; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; FA, Fanconi anemia.

Retinoic acid inhibited FA fibroblast MMP-2 secretion by 93% at 50 μM (data not shown).

Discussion

Elevated MMP levels correlate with tumor progression and metastasis, as documented in experimental and clinical studies (5,6). Epanchintsev *et al* reported the overproduction of secretory factors in Fanconi anemia (FA), such as IL-6, IL-8, MMP-2 and MMP-9 and that overexpression of these secretory factors promoted the proliferation, migration and invasion of surrounding tumor cells (3). Thus, knowledge of MMP regulation is of importance for developing therapeutic strategies for FA. Extracellular factors, including cytokines, growth factors, inducers and inhibitors, have been implicated in the regulation of MMP expression in different types of tumor cells (11,12).

In the present study, we compared MMP secretion patterns by cytokines, PMA and LPS in FA immortalized cell lines. In addition, we investigated the effect of inhibitors doxycycline, EGCG, nutrient mixture (NM) and others, such as dexamethasone, retinoic acid and agents that affect transcription and translation levels, such as actinomycin-D.

Among the inducers and cytokines, PMA treatment had a moderate stimulatory effect on MMP-2 and strong stimulation of MMP-9 secretion, TNFα had a negligible effect on MMP-2 and a significant stimulatory dose-dependent effect on MMP-9, IL-1β had slight stimulation on MMP-2 at 1 and 10 ng/ml and significant stimulatory dose-dependent effects on MMP-9, and LPS showed a moderate stimulatory effect on MMP-2 secretion below 50 μM and an inhibitory effect at 50 and 100 μM and no effect on MMP-9.

Among the chemical inhibitors, doxycycline downregulated the secretion of FA fibroblast MMP-2 and MMP-9 in a dose-dependent manner, with virtual total blockage of MMP-2 at 100 μM and of MMP-9 at 50 μM. In contrast, actinomycin-D had a slight inhibitory effect on MMP-2 and a strong stimulatory effect on MMP-9 secretion. Dexamethasone had a potent inhibitory effect on MMP-2.

Among the natural inhibitors, EGCG downregulated the expression of MMP-2 and PMA induced MMP-9 expression in a dose-dependent manner with virtual total blockage of both at 100 μM. Similarly, NM showed dose-dependent inhibition of MMP-2 and MMP-9 expression in PMA-treated cells with virtual total blockage of both at 500 μg/ml. Retinoic acid strongly inhibited FA fibroblast MMP-2 secretion.

NM, which contains micronutrients such as lysine, proline, ascorbic acid, and green tea extract, has demonstrated anti-tumor and anti-invasive potential *in vivo* and *in vitro* (13). The usage of combinations of micronutrients expands metabolic targets mediated by different pathways, and thus maximizes the biological impact with lower doses of components. For example, a previous comparative study on the effects of NM and its components such as green tea extract and EGCG on the inhibition of MMP-2 and MMP-9 secretion of different cancer cell lines with varying MMP secretion patterns, revealed the superior potency of NM over green tea extract and EGCG at equivalent doses (14).

We designed NM by selecting nutrients that act on critical physiological targets in cancer progression and metastasis, as documented in both clinical and experimental studies. Adequate levels of ascorbic acid, lysine and proline are essential for supporting proper synthesis and hydroxylation of collagen fibers to optimize ECM structure. In addition, lysine contributes to ECM stability as a natural inhibitor of plasmin-induced proteolysis (15,16). Manganese and copper also contribute to collagen formation. Green tea extract has been shown to be potent in modulating cancer cell growth, metastasis, angiogenesis and other aspects of cancer progression (17-21). *N*-acetyl cysteine and selenium have been documented to suppress tumor cell MMP-9 and invasive activities, in addition to migration of endothelial cells through the ECM (22-24). Ascorbic acid has been documented to modulate cancer cell and tumor growth as well as to prevent

metastasis (25-30) and low levels of ascorbic acid are found in cancer patients (31,32). Low levels of arginine limit NO production, an inducer of apoptosis (33).

In conclusion, our results demonstrated that cytokines, mitogens and inhibitors modulated FA fibroblast MMP-2 and MMP-9 secretion, suggesting the clinical use of MMP inhibitors, particularly potent and non-toxic ones such as NM and its component EGCG in the management of FA cancers.

Acknowledgements

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