Research Article

In Vitro Effect of Cytokines, Inducers, and Inhibitors on the Secretion of MMP-2 and MMP-9 in Hepatocarcinoma Cell Line SK-Hep-1

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Abstract

The prognosis of hepatocellular carcinoma (HCC) remains dismal despite any treatment. Matrix metalloproteinases (MMPs) have been researched for their role in tumor invasion and metastasis. Various cytokines, mitogens, growth factors, inducers, and inhibitors control MMP activities. In this article, we investigated the roles of these in the regulation of MMP-2, -9 secretions in HCC. Human HCC SK-Hep-1 was grown in appropriate media. At near confluence, the cells were washed with phosphate-buffered saline and incubated in serum-free media with PMA; TNF-α, IL-1β; lipopolysaccharide; epigallocatechin gallate (EGCG) and doxycycline (Dox) at various doses with and without PMA; a nutrient mixture (NM) containing lysine, proline, ascorbic acid, and EGCG with and without PMA at; and actinomycin D and cycloheximide at different doses. After 24 hours, the media were removed and analyzed. SK-Hep-1 expressed bands corresponding to MMP-2 and MMP-9. TNF-α showed an insignificant effect on MMP-2 at doses below 25 at which dose MMP-2 was virtually blocked and a moderate dose-dependent effect on MMP-9. Interleukin-1β demonstrated an insignificant effect on MMP-2 at doses below 25 at which dose MMP-2 was completely blocked and enhanced effects on MMP-9. Lipopolysaccharide showed dose-dependent inhibition of MMP-2 and MMP-9. EGCG, Dox, and NM, without and with PMA, downregulated the expression of MMP-2 and MMP-9. Actinomycin D and cycloheximide also had dose-dependent inhibitory effects on MMPs. Our results showed that cytokines, mitogens, and inhibitors modulated SK-Hep-1 MMP-2 and MMP-9 secretion, suggesting the clinical use of especially potent and nontoxic MMP inhibitor as the NM in management of HCC.

Keywords
matrix metalloproteinases, HCC SK-Hep-1, cytokines, inducers, inhibitors

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Introduction

Despite advances in clinical study of hepatocellular carcinoma (HCC), its incidence continues to increase, with more than 700 000 people diagnosed annually with this cancer worldwide.[1] It is the leading cause of death worldwide and accounts for more than 600 000 deaths every year.[1] The American Cancer Society's estimates for primary HCC and intrahepatic bile duct cancer in the United States for 2017 are the following: 40 710 new cases (29 200 in men and 11 510 in women) and 28 920 deaths (19 610 men and 9310 women).[1] The most prevalent causes of death in patients with HCC include uncontrolled metastasis and recurrence. In recent years, efforts have been focused on exploring many molecular markers related to invasion, metastasis, recurrence, and survival in HCC. Among these factors, the matrix metalloproteinases (MMPs) and the plasminogen activation system play crucial roles in cancer invasion and metastasis.

Matrix metalloproteinases, a family of zinc- and calcium-dependent proteolytic enzymes, are able to degrade connective tissue, among other substrates, such as basement membrane collagen, and have been associated with

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cancer metastasis and tumor angiogenesis. The gelatinases, especially MMP-9 and MMP-2, play a key role in degradation of collagen type IV, a main component of the basement membranes.[2-4] These gelatinases are expressed in HCC cells and are associated with progression and invasion of these tumors.[5-8] For example, Guo et al noted positive correlation of MMP-9, MMP-2, and vascular endothelial growth factor (VEGF) expression with recurrence of HCC.[9] MMP activity is modulated by environmental influences from surrounding stroma cells, extracellular matrix (ECM) proteins, systemic hormones, and other factors.[10] Inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, have been shown to play significant roles in HCC progression. Pro-inflammatory IL-1β was shown to be elevated in HCC patients compared with healthy individuals.[11] TNF-α expression was elevated in HCC patients, especially those with recurrence.[11] Porta et al demonstrated the overproduction of secretory factors such as IL-6 in HCC.[12]

Rath and Pauling postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, modulate tumor growth and expansion.[13] These nutrients can exercise their antitumor effect by protecting integrity of connective tissue surrounding cancer cells through inhibition of its degradation (MMP-2 and MMP-9) and their necessary role in collagen synthesis. These 2 processes are essential for a tumor encapsulating effect.

Based on this concept, we developed a nutrient mixture (NM) containing lysine, proline, ascorbic acid, green tea extract, and other micronutrients, with the aim to inhibit cancer development and its spread by targeting critical physiological factors in cancer progression and metastasis, including ECM integrity and MMP activity.[14]

Different cancer cell types have special abilities to regulate the secretion of MMPs in response to various synthetic and natural compounds, which consequently has an effect on ECM integrity. This study is a part of an investigation on the effects of select cytokines, inducers, and inhibitors on MMP-2 and MMP-9 secretion by various cancer cell types. Here we studied the in vitro effects of natural and synthetic agents applied in cancer research on MMP-2 and MMP-9 secretion in HCC SK-Hep-1 cell line.

Methods and Materials

Materials

Human hepatoma cell line SK-Hep-1 was obtained from ATCC (American Type Culture Collection, Rockville, MD). Antibiotics, penicillin, and fetal bovine serum were obtained from Gibco (BRL, Long Island, NY). Twenty-four-well tissue culture plates were obtained from Costar (Cambridge, MA). Gelatinase zymography was performed in 10% Novex pre-cast sodium dodecyl sulfate (SDS) polyacrylamide gel (Invitrogen Inc, Carlsbad, CA) with 0.1% gelatin in nonreducing conditions. IL-1β, TNF-α, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), doxycycline (Dox), and epigallocatechin gallate (EGCG) were purchased from Sigma (St. Louis, MO). The NM manufactured by VitaTech (Hayward, CA) was composed of the following ingredients in the relative amounts indicated: vitamin C (as ascorbic acid and as magnesium, calcium, 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, and manganese 1 mg. All other reagents used were of high quality and were obtained from Sigma, unless otherwise indicated.

Cell Culture

SK-Hep-1 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were plated at a density of 1×10^5 cells/mL and grown to confluency in a humidified atmosphere at 5% carbon dioxide at 37°C. Serum-supplemented media were removed and the cell monolayer was washed once with phosphate-buffered saline 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; Dox (10, 25, 50, and 100 μM) without and with PMA 100 ng/mL; NM (10, 50, 100, 500, and 1000 μg/mL) with PMA 100 ng/mL; and 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 minutes at 3000 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. On 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 nonreducing 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

Table 1. Effect of Inducers on Hepatocellular Carcinoma SK-Hep-1 MMP-2 and MMP-9 Secretion.

	MMP-2	MMP-9
PMA (ng/mL)		
Control	100%	100%
10	57.3%	72.2%
25	98.0%	145.8%
50	98.5%	205.9%
100	49.0%	172.5%
TNF-α (ng/mL)		
Control	100%	100%
0.1	83.9%	176.9%
1	91.9%	334.2%
10	123.9%	357.9%
25	3.3%	295.6%
IL-1β (ng/mL)		
Control	100%	100%
0.1	111.3%	179.2%
1	99.1%	221.6%
10	96.6%	242.1%
25	0%	171.5%
LPS (μg/mL)		
Control	100%	100%
10	83%	60.7%
25	43.3%	53.2%
50	73.8%	58.5%
100	64.2%	40.1%

Abbreviations: MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide.

before electrophoresis. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 minutes 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 minutes 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), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi).

Statistical Analysis

The experiments were conducted one time. We took equal volumes from each well and mixed them together to use for analysis. The data from individual wells were not documented; therefore, specific statistical analysis was not conducted.

Results

Inducers and Cytokines

Hepatocellular carcinoma SK-Hep-1 expressed gelatinase zymography bands corresponding to MMP-2 and MMP-9. Table 1 shows the quantitative densitometry results from the effects of PMA, TNF-α, IL-1β, and LPS on MMP-2 and MMP-9 expression in SK-Hep-1 cells.

Effect of PMA on HCC SK-Hep-1 Secretion of MMPs

On gelatinase zymography, SK-Hep-1 demonstrated slight secretion band for MMP-2 and strong expression of MMP-9. PMA treatment had no significant effect on secretion of MMP-2 (linear trend $R^2 = 0.146$) but strongly stimulated the secretion of MMP-9 and MMP-9 dimer in a dose-dependent manner (linear trends $R^2 = 0.668$ and 0.736, respectively). As such, at 100 ng/mL, the MMP-9 increased by 173% of control and MMP-9 dimer secretion was 100× higher than observed at 10 ng/mL (see Figure 1).

Effect of TNF-α on HCC SK-Hep-1 Secretion of MMPs

Tumor necrosis factor-α showed insignificant effect on MMP-2 secretion when used below 10 ng/mL ($R^2 = 0.281$); however, its secretion was blocked by 97% at 25 ng/mL. TNF-α had a significant stimulatory dose-dependent effect on MMP-9 ($R^2 = 0.674$). The MMP-9 secretion was 334% and 358% higher at 1 and 10 ng/mL, respectively, compared with control and 296% at 25 ng/mL, as shown in Figure 2.

Effect of IL-1β on HCC SK-Hep-1 Secretion of MMPs

Interleukin-1β showed an insignificant effect on MMP-2 secretion when applied below 10 ng/mL ($R^2 = 0.126$), with a complete inhibition at 25 ng/mL. On the other hand, IL-1β had significant stimulatory effect on MMP-9 ($R^2 = 0.353$), reaching 242% of control at 10 ng/mL. At a higher concentration of 25 ng/mL, this effect was slightly suppressed to 172% of control, as shown in Figure 3.

Effect of LPS on HCC SK-Hep-1 Secretion of MMPs

The results in Figure 4 show that, in general, LPS had inhibitory effect on MMP-2 ($R^2 = 0.364$), secretion and

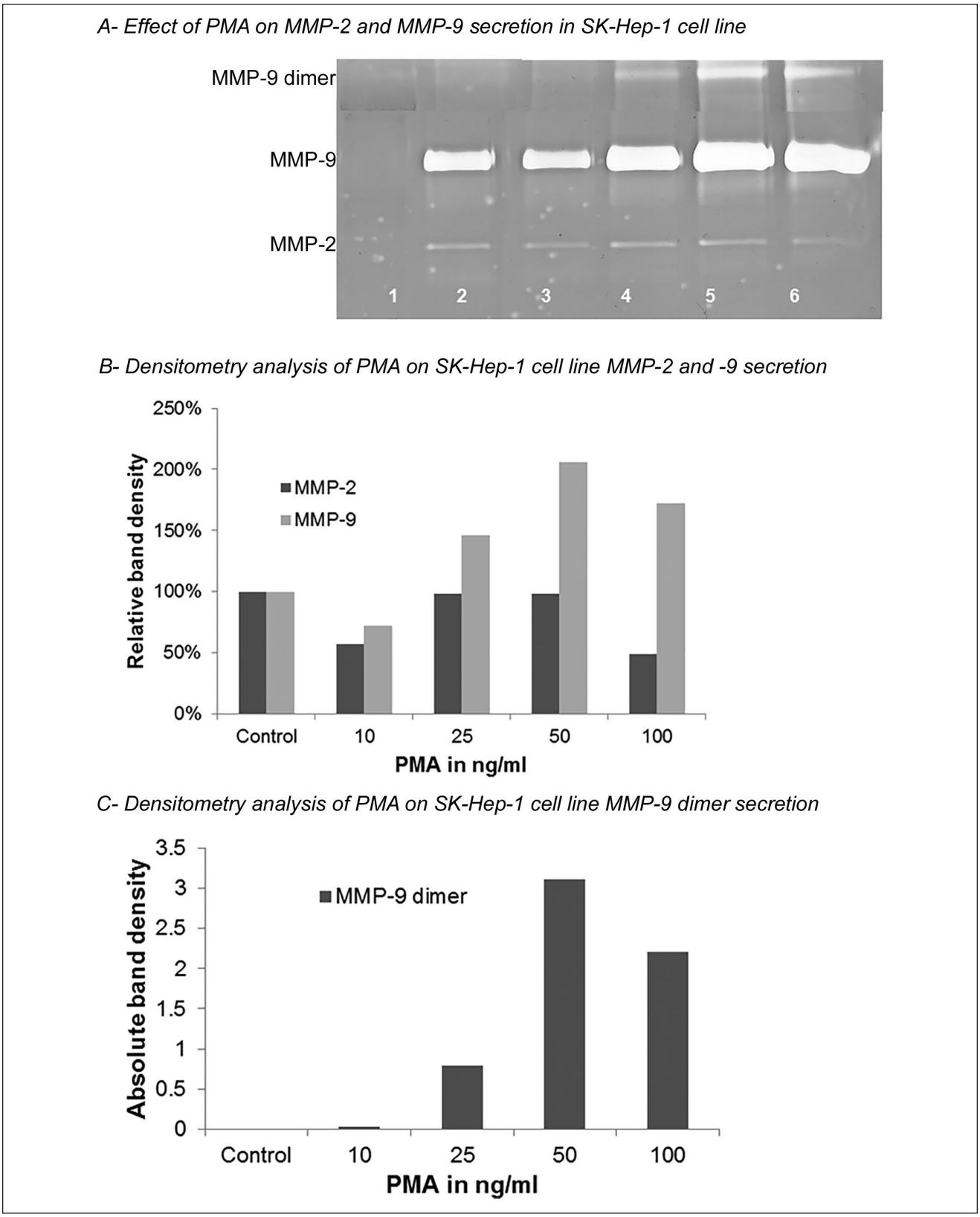

Figure 1. Effect of phorbol 12-myristate 13-acetate (PMA) on matrix metalloproteinase (MMP)-2 and MMP-9 expression in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymogram (A) and densitometry analysis (B) of SK-Hep-1 MMP-2 and MMP-9 expressions and (C) of SK-Hep-1 MMP-9 dimer expression.
1, markers; 2, control; 3-6, 10, 25, 50, and 100 ng/mL PMA.

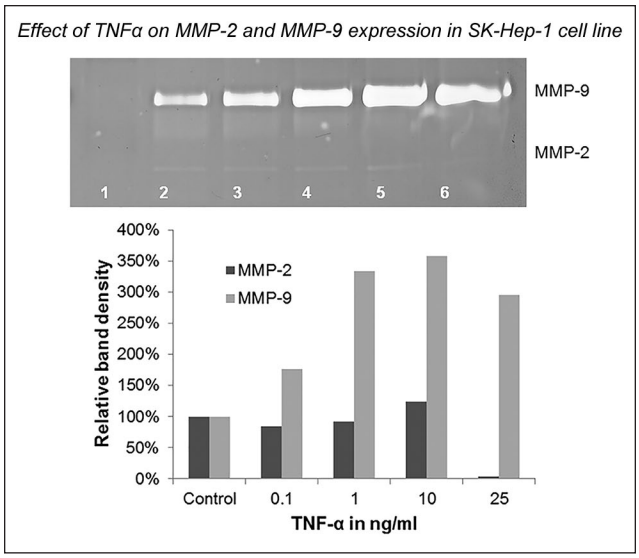

Figure 2. Effect of tumor necrosis factor (TNF)-α on matrix metalloproteinase (MMP)-2 and MMP-9 secretions in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymogram (A) and densitometry analysis (B) of SK-Hep-1 MMP-2 and MMP-9 expressions.
1, markers; 2, control; 3-6, TNF-α 0.1, 1, 10, and 25 ng/mL.

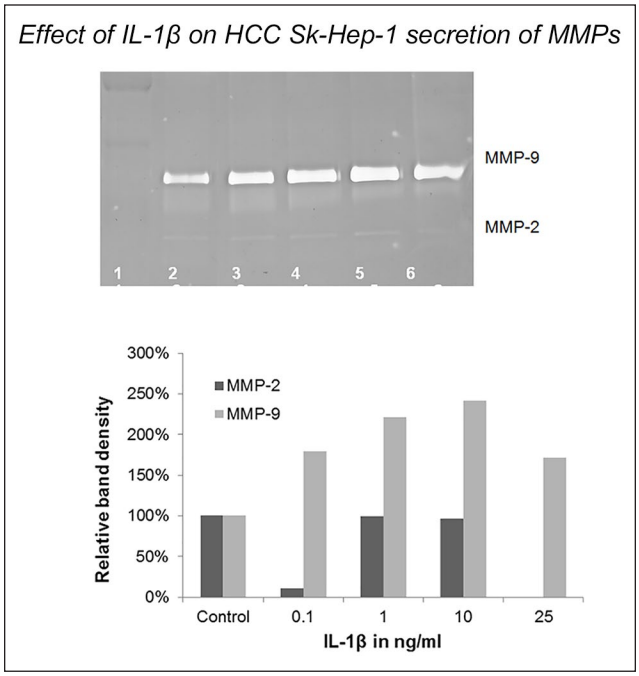

Figure 3. Effect of interleukin (IL)-1β on matrix metalloproteinase (MMP)-2 and MMP-9 secretions in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymogram (A) and densitometry analysis (B) of SK-Hep-1 MMP-2 and MMP-9 expressions.
1, markers; 2, control; 3-6, IL-1β 0.1, 1, 10, and 25 ng/mL.

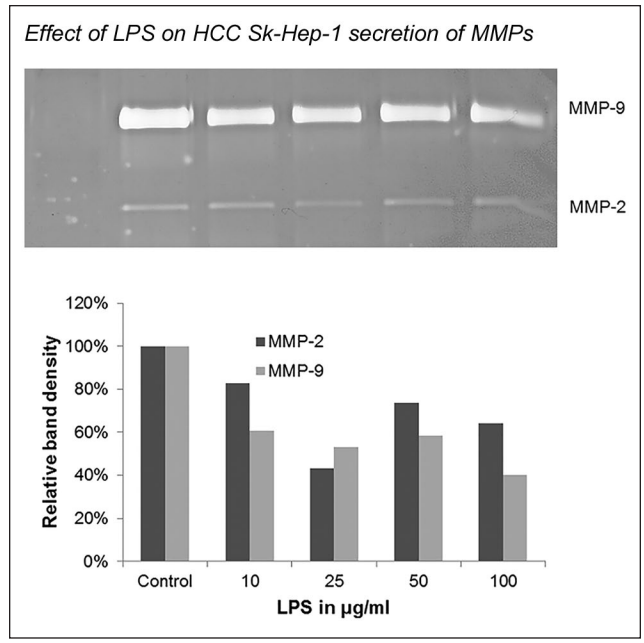

Figure 4. Effect of LPS (lipopolysaccharide) on matrix metalloproteinase (MMP)-2 and MMP-9 secretions in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymogram (A) and densitometry analysis (B) of SK-Hep-1 MMP-2.
1, markers; 2, control; 3-6, interleukin-1β, 0.1, 1, 10, and 25 ng/mL.

strong inhibitory dose-dependent effect on MMP-9 (R^2 = 0.739), reaching 40% of control at 100 µg/mL.

Chemical Inhibitors

Table 2 shows the quantitative densitometry results of the effects of chemical inhibitors Dox, actinomycin-D, and cycloheximide on MMP-2 and MMP-9 expression in HCC SK-Hep-1 cell line.

Figure 5 shows that Dox slightly inhibited MMP-9 secretion at dose range of 10 to 50 µM and demonstrated strong inhibition (65% of control) of secretion at 100 µM (R^2 = 0.460). In the presence of PMA 100 ng/mL, Dox decreased the expression of MMP-9 monomer and dimer in a dose-dependent manner, with 73% decrease in MMP-9 monomer at 100 µM (R^2 = 0.710) and total block of MMP-9 dimer at 100 µM (R^2 = 0.744; as shown in Figure 6). Actinomycin D had a dose-dependent inhibitory effect on MMP-2 secretion (R^2 = 0.999) with 62% inhibition at 4 µM and dose-dependent inhibition of MMP-9 (R^2 =0.912) with 49% inhibition at 4 µM. Cycloheximide had dose-dependent inhibitory effect on MMP-2 (R^2 = 0.932) with total block at 4 µM and dose-dependent inhibition of MMP-9 (R^2 =0.988) with 63% inhibition at 4 µM.

Table 2. Effect of Inhibitors on Hepatocellular Carcinoma SK-Hep-1 MMP-2 and MMP-9 Secretion.

	Untreated	PMA 100 ng/mL–Treated	
	MMP-9	MMP-9	MMP-9 Dimer
Doxycycline (μM)			
Control	100%	100%	100%
10	76.3%	88.2%	93.4%
25	80.5%	87.2%	93.8%
50	95.8%	81.6%	66.5%
100	34.7%	27.0%	0%
EGCG (μM)			
Control	100%	100%	
10	71.6%	74.4%	
25	54.3%	59.5%	
50	42.5%	55.2%	
100	17.8%	53.7%	

	Untreated		PMA 100 ng/mL–Treated	
	MMP-2	MMP-9	MMP-2	MMP-9
NM (μg/mL)				
Control	100%	100%	100%	100%
10	118.6%	107.1%	105.7%	117.6%
50	74.8%	67.4%	98.1%	109.2%
100	35.2%	36.1%	76.2%	95.7%
500	2.2%	6.8%	32.1%	34.7%
1000	1%	1%	1%	1%
Actinomycin D (μM)				
Control	100%	100%		
2	67.2%	62.4%		
4	37.9%	51.1%		
Cyclohexamide				
Control	100%	100%		
2	73.4%	62.5%		
4	0%	37.1%		

Abbreviations: MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; EGCG, epigallocatechin gallate; NM, nutrient mixture.

Natural Inhibitors

Table 2 shows quantitative densitometry results of the effects of natural compounds EGCG and the NM on MMP-2 and MMP-9 secretion in HCC SK-Hep-1.

Figure 7A shows that EGCG down regulated the secretion of MMP-9 by SK-Hep-1 cells in a dose-dependent manner, achieving its 82% block at 100 μM ($R^2 = 0.981$). EGCG also demonstrated its inhibitory effect on MMP-9 secretion in the presence of PMA (100 ng/mL) in a dose-dependent manner with 46% block at 100 μM ($R^2 = 0.831$), as shown in Figure 7B. NM inhibited secretion of MMP-2 and MMP-9 by uninduced SK-Hep-1 cells in a dose-dependent manner, with virtual total block of both at 1000 μg/mL, showing linear trends $R^2 = 0.889$ and $R^2 = 0.934$, respectively (see Figure 8A). In PMA-treated cells (shown in Figure 8B), the NM displayed dose-dependent inhibition of MMP-2 and MMP-9 secretion with its virtual total block of both enzymes at 1000 μg/mL, and with linear trends $R^2 = 0.846$ and $R^2 = 0.736$, respectively.

Discussion

Elevated MMP levels, especially MMP-2 and MMP-9, are prognostic for high metastatic potential and poor survival in HCC, as documented in clinical studies.[5,6,8,9] Määtä et al[6] reported elevated MMP-2 and MMP-9 levels in malignant tissue with latent and active MMP-2 levels primarily located in tumor stroma and MMP-9 in neoplastic epithelial cells. Furthermore, elevated MMP-2 and MMP-9 mRNA correlated with poorer survival of patients. In assessing MMP-9 and MMP-2 levels immunohistochemically in HCC tissue microarrays from HCC patients who had undergone curative

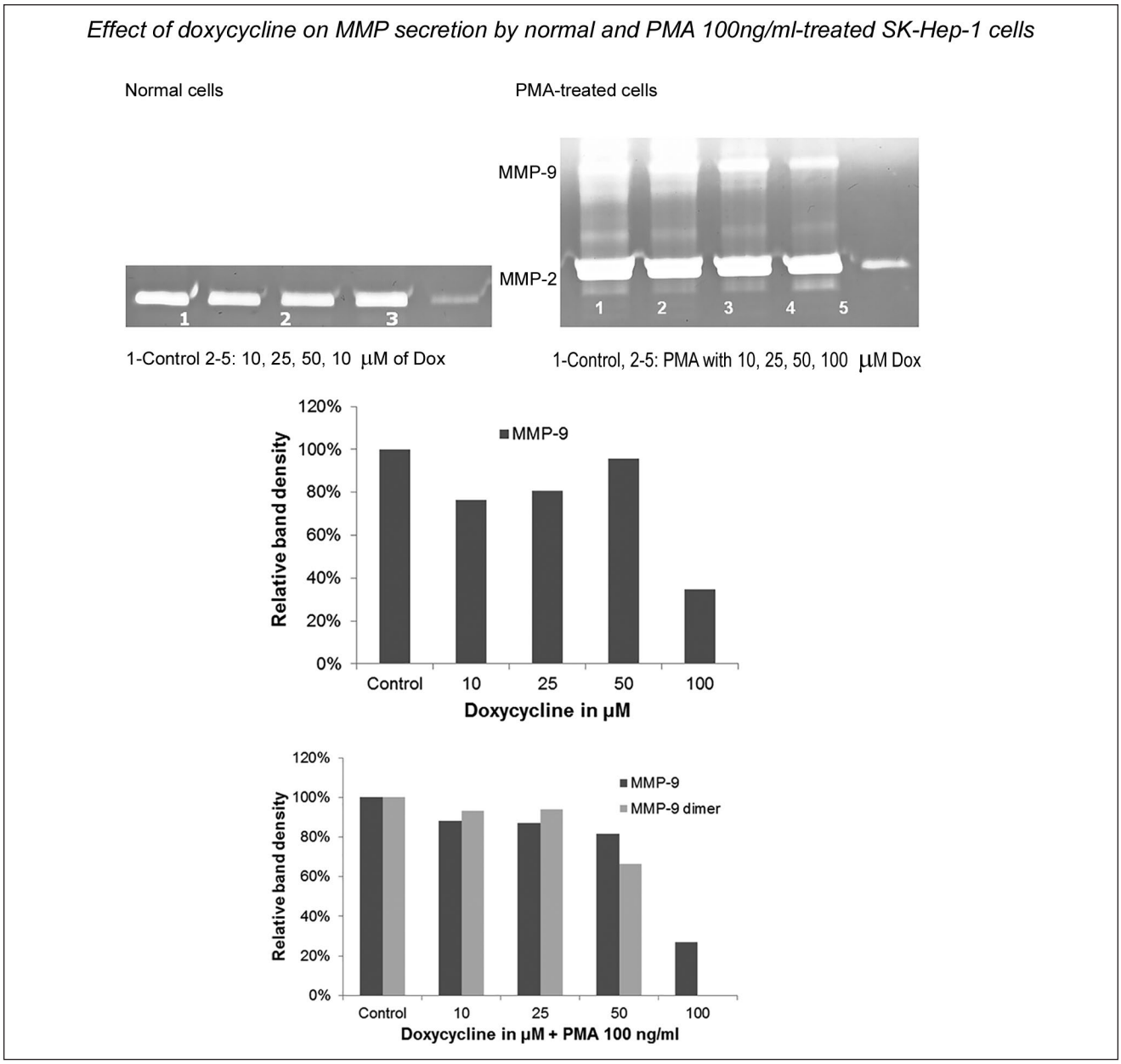

Figure 5. Effect of doxycycline on matrix metalloproteinase (MMP)-2 and MMP-9 secretion by normal and phorbol 12-myristate 13-acetate (PMA) 100 ng/mL-treated cells in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymograms (A) of normal SK-Hep-1 cells, (B) PMA-treated SK-Hep-1 cells and densitometry analyses (C) of normal SK-Hep-1 cells, and (D) PMA-treated SK-Hep-1 cells.
1, markers; 2, control; 2-5, doxycycline 10, 25, 50, and 100 μM.

resection, high expression of MMP-9 was associated with both earlier recurrence and poor overall survival, whereas high expression of MMP-2 was only correlated with shorter time to recurrence.[15] Sakamoto et al[5] found that overexpression of MMP-9 mRNA in tissues of HCC patients correlated with growth of small HCC. Guo et al observed that overexpression of MMP-9 and MMP-2 and high VEGF levels in patients with HCC positively correlated with recurrence.[9] Jiang et al[8] found that high MMP-9 expression correlated with recurrence or metastasis of HCC in patients post hepatectomy. Thus, knowledge of MMP regulation is important for developing effective therapeutic strategies for hepatic cancers. It has been shown that inflammatory cytokines, such as IL-1β and TNF-α, play significant roles in HCC progression. As such, proinflammatory IL-1β was shown to be elevated in HCC patients compared with healthy individu-

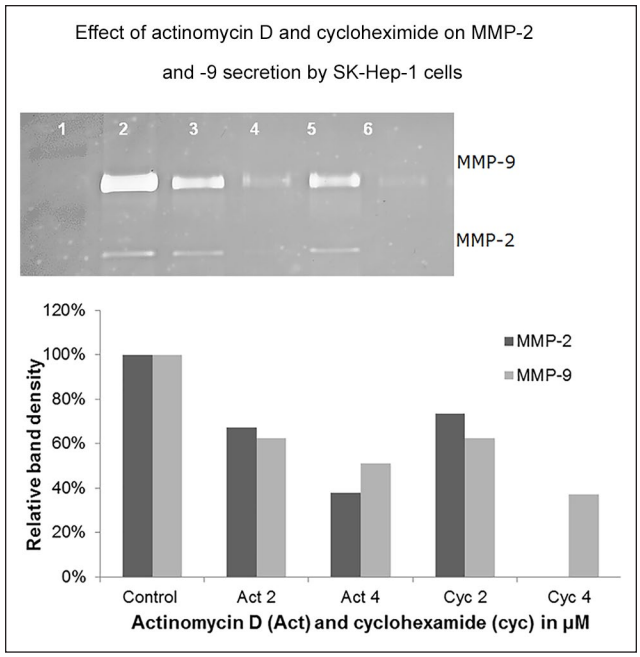

Figure 6. Effect of actinomycin D and cycloheximide on matrix metalloproteinase (MMP)-2 and MMP-9 secretion by normal cells in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymogram (A) of normal SK-Hep-1 cells and densitometry analysis (B) of MMP-2 and MMP-9 expressions.
1, markers; 2, control; 3-4, actinomycin D 2 µM, 4 µM; 5-6, cycloheximide, 2 µM, 4 µM.

als.[11] TNF-α expression was elevated in HCC patients, especially those with recurrence.[11]

In this study, we conducted a systemic evaluation of MMP secretion patterns under influence of pro-inflammatory cytokines, PMA, and LPS in HCC SK-Hep-1. In addition, we investigated the effect of Dox, an antibiotic with pro-apoptotic, anti-inflammatory, and antitumor effects, although in some studies Dox demonstrated pro-tumor activity,[16] as well as protein synthesis inhibitor cycloheximide and actinomycin-D, which is being used as anticancer drug. The effects of these chemical compounds on MMPs secretion were compared with natural components: EGCG from green tea and a mixture of natural compounds (vitamin C, lysine, proline, EGCG, N-acetyl cysteine and others included in NM).

The results showed that none of the tested inducers and cytokines enhanced MMP-2 secretion in HCC SK-Hep-1 cells. Cell exposure to pro-inflammatory PMA and TNF-α resulted in potent inhibition of MMP-2 secretion at 100 ng/mL. However, PMA used at 100 ng/mL concentrations had strong dose-dependent stimulatory effect on MMP-9 secretion up to 172% that of control also TNF-α at 1 to 25 ng/mL concentrations increased MMP-9 secretion by about 300% compared with control. Similarly to PMA and TNF-α, IL-1β did not show significant effect on MMP-2 secretion according to the zymography data. However, its 1.1 ng/mL up to 10 ng/mL concentrations upregulated MMP-9 secretion.

It is interesting that LPS—a pro-inflammatory endotoxin—had decreasing effect on both MMP-2 and MMP-9 secretion in this hepatocarcinoma cell line, which implies its different regulatory pattern.

Among tested inhibitors, chemical compound Dox and natural green tea catechin, the EGCG applied with and without PMA, significantly downregulated the secretion of MMP-9 in SK-Hep-1 cells in a dose-dependent manner. NM applied with and without PMA also showed strong inhibition of both MMP-2 and MMP-9 secretions in SK-Hep-1 in dose-dependent manners. These results correlated with our previous findings in different types of cancer cells.[17,18] Both cycloheximide and actinomycin D inhibited MMP-2 and MMP-9 secretions as observed in other studies.[19]

The strong inhibition of MMP-2 and MMP-9 secretion by natural compounds such as NM and EGCG is of significance in relation to the development of anticancer therapies not associated with negative side effects, characteristic for conventional cancer treatments. This specific composition (NM) has demonstrated anticancer efficacy in various in vitro and in vivo studies in different cell types by affecting key mechanisms of malignancy, including secretion of MMPs.[14] NM was formulated by selecting nutrients that act on critical physiological targets in cancer progression and metastasis. Ascorbic acid, lysine, proline, copper, manganese, and N-acetyl cysteine are essential for optimizing synthesis, integrity, and stability of connective tissue, contributing to tumor encapsulation and preventing cancer cell invasion.[20-24] Ascorbic acid has been documented to modulate cancer cell and tumor growth through its direct pro-oxidant effects and curb metastasis through its encapsulating effects.[25-29] Low levels of ascorbic acid are found in cancer patients.[30,31] Green tea extract has been shown to potently modulate cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression.[32-38]

Comparing the effect of EGCG alone and its equivalent dose in combination with other micronutrients in NM in both uninduced and PMA-treated SK-Hep-1 cells demonstrated the superior inhibitory action of NM over EGCG alone on secretion of MMP-9. NM at 500 µg/mL contains 38.3 µg/mL EGCG. Our results showed that EGCG used alone at 100 µM (equivalent to 45.8 µg/mL), in both uninduced and PMA-induced SK-Hep-1 cells, could inhibit MMP-9 secretion by 82% and 46%, respectively. However, 38.3 µg/mL EGCG contained in 500 µg/mL concentration of NM was more effective than EGCG alone (even at slightly higher amount of 45.8 mg/mL) and resulted in 93% inhibition of MMP-9 secretion in uninduced cells and 65% inhibition in PMA-induced SK-Hep-1 cells. MMP inhibitory effect of NM has persisted in the presence of PMA.

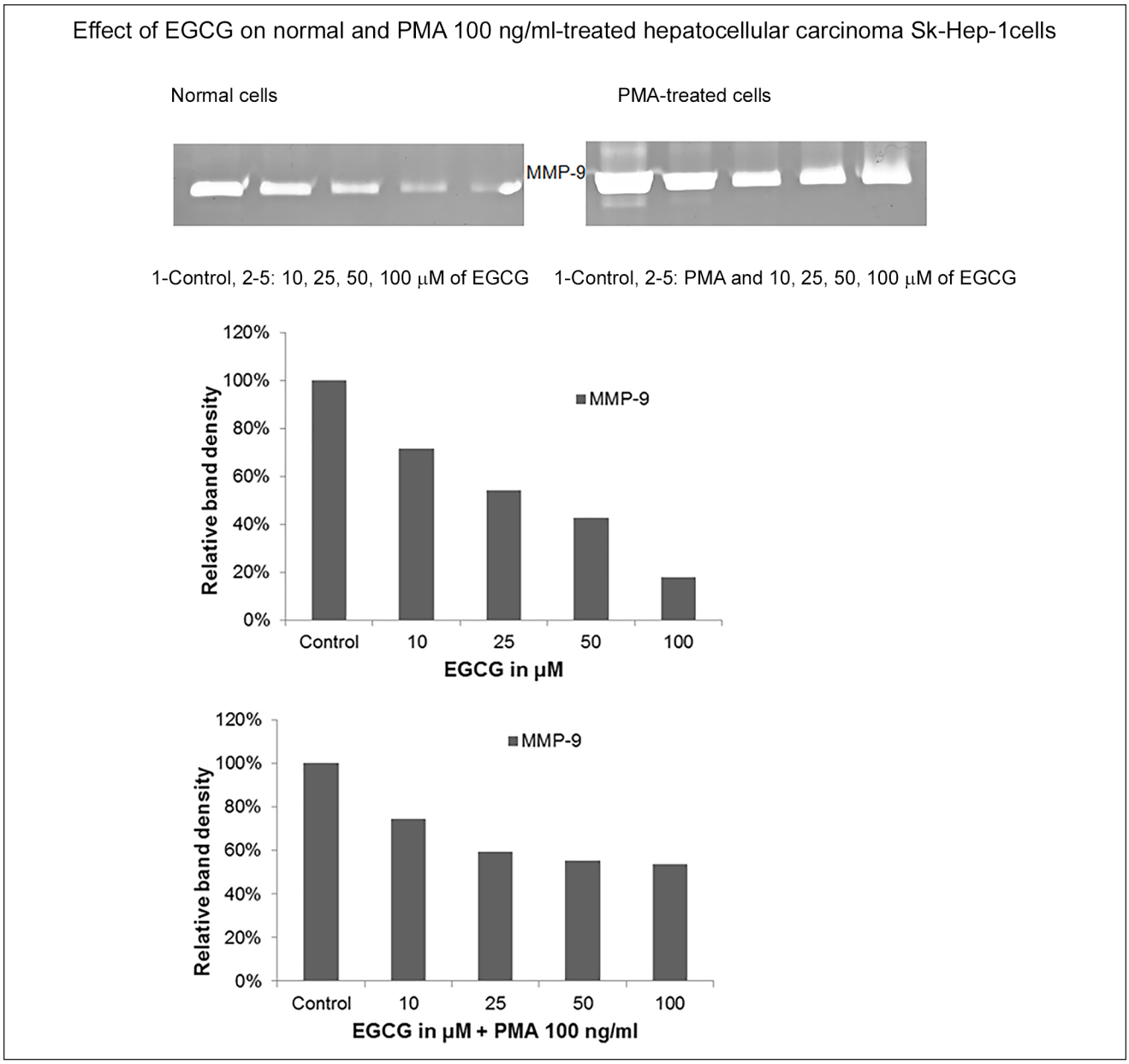

Figure 7. Effect of epigallocatechin gallate (EGCG) on matrix metalloproteinase (MMP)-2 and MMP-9 secretions by normal and phorbol 12-myristate 13-acetate (PMA) 100 ng/mL-treated cells in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymograms (A) of normal SK-Hep-1 cells, (B) PMA-treated SK-Hep-1 cells and densitometry analyses (C) of normal SK-Hep-1 cells, and (D) PMA-treated SK-Hep-1 cells.
1, markers; 2, control; 2-5, EGCG 10, 25, 50, and 100 μM.

Some limitations of this study include the single replication of the experiment. Although this is not common, such a method is also employed by other researchers studying the role of MMPs in other biological processes.[39,40] While our experiments were conducted only once in this case, our previous research has shown that the NM has a significant anticancer potential and effectively targets multiple mechanisms of cancer metastasis in various types of cancer.[18,41] Hence, we think that this study can be looked at as additional evidence. Another limitation of this study could possibly be the clinical significance and correlation of effective dose of NM in patients with hepatocarcinoma. Here we have studied the doses from 10 μg/mL to 1000 μg/mL of NM; however, the highest concentration of 1000 μg/mL may not be clinically relevant. In animal studies, we use a diet with 0.5% NM concentration. Comparison of different dietary intakes of NM

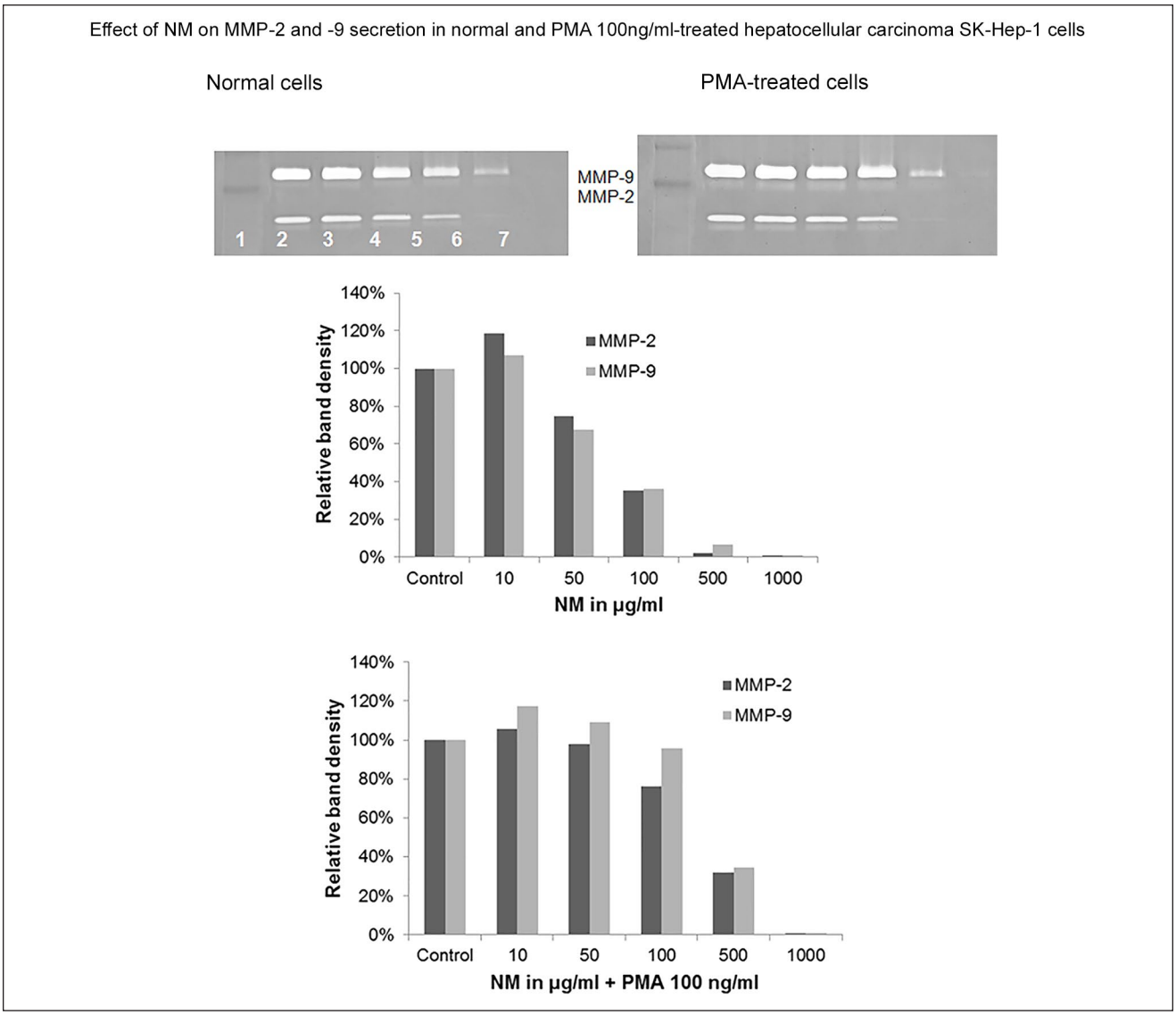

Figure 8. Effect of nutrient mixture (NM) on matrix metalloproteinase (MMP)-2 and MMP-9 secretions by normal and phorbol 12-myristate 13-acetate (PMA) 100 ng/mL-treated cells in hepatocellular carcinoma SK-Hep-1 cell line. Gelatinase zymograms (A) of normal SK-Hep-1 cells, (B) PMA-treated SK-Hep-1 cells and densitometry analyses (C) of normal SK-Hep-1 cells, and (D) PMA-treated SK-Hep-1 cells.
1, markers; 2, control; 3-7, NM 10, 50, 100, 500, and 1000 µg/mL.

showed that at 0.5% the clinical efficacy reaches a plateau as 1% NM in a rodent diet does not show any additional anticancer effects. Taking into account that a mouse eats about 20 g of feed daily this would correspond to NM intake of 100 mg. NM is composed of different nutrients with different rates of absorption and assimilation; however, the entire composition provides anticancer effects. The exact human dose of NM to be applied in cases of hepatocarcinoma should be tested in clinical trials.

Use of nutrient combinations in controlling various cellular processes has advantages over a single nutrient or a chemical compound. First, that chemical compounds such as tested here have a very low margin of safety and can only be applied at very low doses. Second, properly selected multinutrient combination can demonstrate pleiotropic cellular effects important in targeting metabolic diversity of cancer. Even more, in such a mixture as NM we could achieve better efficacy in inhibiting MMPs secretion at lower doses of its individual components, as illustrated with EGCG. Not all mixtures are equally effective and sometimes small changes in their composition have a large impact on the final efficacy.

Conclusion

In conclusion, our results showed that cytokines, mitogens, and chemical and natural inhibitors modulate MMP-2 and MMP-9 secretion in SK-Hep-1 cells, suggesting clinical potential of applying potent and nontoxic NM and/or EGCG in management of HCC cancers.

Declaration of Conflicting Interests

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