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Anti-inflammatory Property of β-D-Mannuronic Acid (M2000) on Expression and Activity of Matrix Metalloproteinase-2 and -9 through CD147 Molecule in Phorbol Myristate Acetate-differentiated THP-1 Cells

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ABSTRACT

The aim of this study was to evaluate the effects of M2000, a novel non-steroidal anti-inflammatory drug (NSAID) with immunosuppressive property and without gastro-nephrotoxicitic effects on matrix metalloproteinases (MMP)-2 and (MMP)-9 in phorbol myristate acetate (PMA)-differentiated THP-1 cells. Gene expression and activity of MMP-2 and MMP-9 are inhibited respectively by the tissue inhibitor of matrix metalloproteinase (TIMP)-2 and (TIMP)-1 and are induced by extracellular matrix metalloproteinase inducer (CD147/EMMPRIN).

In this study, real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to determine gene expression of MMP-2, MMP-9, TIMP-1, and TIMP-2. Flow cytometry and zymography were applied to determine cellular surface expression of CD147 and activity of MMP-2 and MMP-9, respectively.

Our results showed that treatment of THP-1 cells with high concentration (25 μg/mL) of M2000 significantly decreased the cellular surface expression of CD147 ($p<0.05$) and the gene expression of MMP-2, MMP-9 and TIMP-1 ($p<0.05$), and inhibited the gelatinolytic activity of MMP-2 and MMP-9 ($p<0.05$).

According to our results, M2000 can reduce inflammation through inhibition of the cellular surface expression of CD147 and decrease the gene expression and gelatinolytic activity of MMP-2 and MMP-9 in PMA-differentiated THP-1 cells.

Keywords: CD147; Mannuronic acid; Matrix metalloproteinases; Tissue inhibitor of matrix metalloproteinase

INTRODUCTION

Matrix metalloproteinases (MMPs) or matrixins are a family of proteolytic enzymes with zinc-dependent function at neutral Ph.1 They have key roles in biological processes such as morphogenesis, wound

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healing, tissue repair and remodeling in response to injury, and also are involved in the progression of diseases such as arthritis, cancer, cardiovascular disease, and chronic tissue ulcers through degradation of the components of extracellular matrix (ECM).[2] MMPs are classified into six groups: collagenases, gelatinases, stromelysines, matrilysins, membrane type-MMPs, and other MMPs.[1] The increased expression and activity of MMPs are seen in the various human inflammatory diseases.[3] The role of MMPs in inflammation is elicited through the regulation of permeability of epithelial and endothelial barriers, inflammatory cytokines and chemokines activation, and creation of the chemokine gradient.[3] Expression and activity of MMPs are regulated by TIMPs and CD147.

Gelatinases (MMP-2 and MMP-9) are two important members of this family that are involved in diseases such as rheumatoid arthritis, systemic lupus erythematosus,[4] multiple sclerosis,[5] glomerulonephritis,[6] inflammatory bowel disease,[7] diabetes,[8] and cancers.[9,10]

CD147 is a transmembrane glycoprotein and a member of the immunoglobulin superfamily.[11] CD147 is expressed on the surface of different types of cells, including hematopoietic cells, epithelial cells, endothelial cells, immune cells, smooth muscle cells, and tumor cells.[12] CD147 performs various roles through glycosylation in different sites of the extracellular domain, and through interaction with different ligands including cyclophilins, caveolin-1, monocarboxylate transporters (MCT-1, 3, 4), integrins, E-selectin, S100A9, CD98, CD44, and CD147 itself.[12] Due to its role in inducing the production of several matrix metalloproteinases, CD147 is referred to as "extracellular matrix metalloproteinase inducer (EMMPRIN)".[13]

Tissue inhibitors of matrix metalloproteinases (TIMPs) are other regulators of MMPs. The family of TIMPs has four members (TIMP-1, TIMP-2, TIMP-3, and TIMP-4). Each of them inhibits a wide range of MMPs' activities;[2] however, TIMP-1 and TIMP-2 are preferably inhibitors for MMP-9 and MMP-2, respectively.[14] Imbalances between TIMP-1/2 and MMP-9/2 have been observed in different diseases such as rheumatoid arthritis,[15] multiple sclerosis,[16] and various cancers.[17-19]

β-D-mannuronic acid (M2000), patented (DE; 102016113018.4) is a novel non-steroidal anti-inflammatory drug (NSAID) with immunosuppressive property, low molecular weight, no gastro-nephrotoxicity that its therapeutic effects have been reported in experimental models of rheumatoid arthritis (AIA), multiple sclerosis (EAE), nephrosis and immune complex glomerulonephritis (ICG).[20] Moreover, this drug inhibited MMP-2 activity.[21]

In the present study, we investigated the effect of M2000 on MMP-2, MMP-9 gene expression and gelatinolytic activity, TIMP-1, TIMP-2 gene expression and CD147 expression on the cell surface by using a PMA-differentiated THP-1cells.

MATERIALS AND METHODS

This experimental study was approved by the Ethical Committee of Tehran University of Medical Sciences, Tehran, Iran (No. IR.TUMS.REC.1395.2816).

Materials and Reagents

Human monocytic THP-1 cells were obtained from the National Cell Bank of Iran (NCBI). M2000, patented (DE; 102016113018.4) was synthesized in immunology department of Tehran University of Medical Sciences. Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine were purchased from Gibco (Germany). Phorbol 12-myristate 13-acetate (PMA), dimethylsulfoxide (DMSO), Coomassie blue, and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Company (Germany). YTA Total RNA Purification Mini kit was purchased from Yekta Tajhiz Azma Company (Iran). PrimeScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara-bio Company (Japan). Used primers for real-time quantitative reverse transcription qRT-PCR were synthesized by Bioneer Corporation (Germany). Anti-Human CD147 PE-conjugated and Mouse IgG1 K Isotype Control PE-conjugated were purchased from the eBioscience Corporation.

Cell Culture, Differentiation, and Treatment

Human monocytic THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2% *L*-glutamine, and 1% penicillin/streptomycin in culture flasks. Cells were incubated at 37°C in a humidified 5% CO2 atmosphere. For all experiments, cells were plated at a density of 1×10^6 cells/mL into 6-well culture plates in RPMI-1640 with 10% FBS. Then, cells were

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differentiated into macrophage by treatment with PMA (final concentration of 25 ng/mL) for 24 h. Differentiated monocytes to macrophages are adhered to the bottom of cell culture plates and they become flat and amoeboid-shaped. After differentiation, the supernatant was aspirated to remove non-adherent (undifferentiated) cells, and adherent cells were washed with RPMI-1640 medium, and complete medium was added. PMA-differentiated THP-1 cells were treated with approved concentrations of M2000 for clinical trial (5 μg/mL, 25μg/mL), in RPMI-1640 with 10% FBS, and maintained at 37°C in a humidified 5% CO2 incubator for 24 h.

Experimental grouping was as follows:

Group 1: undifferentiated THP-1 cells (negative control)

Group 2: PMA-differentiated THP-1 cells (positive control)

Group 3: PMA-differentiated THP-1 cells were treated with 5 μg/mL of M2000

Group 4: PMA-differentiated THP-1 cells were treated with 25 μg/mL of M2000

Cytotoxicity of M2000

Cytotoxicity of M2000 at different concentrations was evaluated by MTT assay. For this purpose, THP-1 cells were plated at a density of 5×10^4 cells/mL into 96-well culture plates in RPMI-1640 medium with 10% FBS. After differentiation, cells were incubated with different concentration of M2000 (0, 5, 10, 25, 50, 100 and 200 μg/mL) in RPMI-1640 with 10% FBS for 24 h. The medium was replaced by 200 μL of MTT solution (final concentration of 0.5 mg/mL), and incubated for 4 h. Then, MTT solution was aspirated and 200 μL DMSO was added for 5 min to dissolve the formazan blue crystals in the cells. The absorbance value of each well was measured at 570 nm using a Biotech microplate reader.

RNA Extraction and Real-Time Quantitative RT-PCR

THP-1 cells were cultured, differentiated, and treated as previously described. Total RNA was obtained using an YTA Total RNA Purification Mini kit, according to the manufacturer's instructions. RNA concentration and purity were determined from the ratio of absorbance at 260 nm and 280 nm by Nanodrop 2000. cDNA was synthesized from the isolated total RNA, according to the manufacturer's instructions of PrimeScript RT reagent kit. Reverse-transcription master mix containing 5*PrimeScript Buffer (2 μL), PrimeScript RT Enzyme Mix 1 (0.5 μL), and OligoDt Primer 50 μM (0.5 μL) were added to 1μg RNA from each of the samples. The final volume was brought up to 10 μL with nuclease-free water. All components were mixed on ice and then incubated for 15 min at 37 °C to activate the enzyme and 5 s at 85°C to inactivate the enzyme. Then, synthesized cDNA was used for real-time quantitative RT-PCR. Quantification of the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNAs were determined relative to GAPDH by the $2^{-\Delta\Delta CT}$ method. Expression is given as change of mRNA copy number relative to positive control cells. Real-time quantitative RT-PCR components were as follows: cDNA (4 μL), each of reverse and forward primer (2 μL), real MasterMix (10 μL), and nuclease-free water (2 μL). The primers (forward, reverse) for (1):

MMP2 were:
5′- GGCAGTGCAATACCTGAACACC-3′, 5′-GTCTGGGGCAGTCCAAAGAACT - 3′
MMP9 were: 5′- GCACGACGTCTTCCAGTACC-3′, 5′- CAGGATGTCATAGGTCACGTAGC -3′ TIMP-1 were: 5′- TTCTGGCATCCTGTTGTTG CT-3′, 5′-CCTGATGACGAGGTCGGAATT-3′ TIMP-2 were:
5′- TGGAAACGACATTTATGGCAACCC-3′, 5′-CTCCAACGTCCAGCGAGACC-3′; and for GAPDH were: 5′- GAGTCCACTGGCGTCTTCA-3′, 5′-TCCTT GAGGCTGTTGTCAT -3′.

Real time q-PCR was performed using an ABI-7000 Thermal Cycler (Applied Biosystems). The used protocol for all genes was: template pre-denaturation (30 s at 95°C), denaturation (5 s at 95°C), annealing and extension (30 s at 60°C), for 50 cycles. The used protocol for melting curve analysis was: 15 s at 95°C, 1 min at 60°C, and then 15 s at 95°C. Experiments were repeated two times for each sample.

Flow Cytometry

THP-1 cells were cultured, differentiated, and treated as described. Cells were washed with cold phosphate-buffered saline (PBS) on ice to separate from wells. Cell suspensions were divided into two tubes and treated with phycoerythrin-labeled mouse anti-human CD147 and phycoerythrin-labeled mouse IgG1 antibodies as a control separately, and incubated on ice for 45 min. Then, cell surface expression of CD147 was determined by a BD FACS Calibur Flow Cytometer (BectonDickinson, Heidelberg, Germany).

The FACS data were analyzed by Flowjo 7.6.1 software and mean fluorescence intensity (MFI) was obtained for each of the samples.

Zymography

Gelatinolytic activity of MMP-2 and MMP-9 were determined by zymography using 10% sodium dodecyl sulfate (SDS)- polyacrylamide gels with 0.5% (w/v) gelatin. For this purpose, THP-1 cells were differentiated and treated as previously described and cell culture supernatant was collected. The protein concentration of all samples was measured using Bradford test and adjusted to concentration of 10µg. The optimum volume of each sample was mixed with the same volume of 2X non-reducing sample buffer. Each of sample, 20 µL was loaded in each lane without prior boiling. After electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h and incubated for 18-24 h with zymography buffer (1MTris–HCl, 0.876 gr of NaCl, 0.11gr of CaCl2, 0.02 gr of NaN3 and 1 M Zncl2 , pH 7.5) at 37°C in order to digest the gelatin substrate. After washing the gels with water, they were stained for 2 h with 0.5% Coomassie blue in 30% methanol and 10% acetic acid, and destained in a solution of 30% methanol or ethanol, 10% glacial acetic acid, and 60% water. Gelatinolytic activity was characterized as clear bands against the blue stained background. Bands were size-calibrated against a prestained protein ladder (Blue plus III protein marker). Gels were scanned using the Bio Rad GS-800 Calibrated Densitometer and band intensity was determined with Image j 1.46r (Image j software, 1.46r version, National Institutes of Health, USA).

Statistical Analysis

All statistical analysis was performed by GraphPad Prism 6 (GraphPad Software, Inc., version 6.01, USA). Statistical differences among groups were calculated using a one-way analysis of variance (ANOVA) and multiple comparisons were done by the Dunnet adjustment. Statistical difference with $p<0.05$ was considered significant.

RESULTS

Cell differentiation

For all experiments, THP-1 cells were cultured at an initial density of 1×10^6 cells/mL in 6-well culture plates in RPMI-1640 with 10% FBS. To differentiate, all the wells other than the negative control group were treated with PMA (final concentration of 25ng/mL). After 24 h, about 80% of the cells were differentiated into macrophages, which are characterized by being flat and amoeboid in shape, and by adhesion to the bottom of the cell culture plates (Figure 1).

Cytotoxicity of M2000

Effect of M2000 on the viability of PMA-differentiated THP-1 cells was evaluated using the MTT assay. Our results showed no significant changes in cell viability except in the concentration of 1000 µg/mL (Figure 2).

Effect of M2000 on MMP-2 and MMP-9 Gene Expressions

Real-time quantitative RT-PCR indicated that expression of MMP-9 mRNA was markedly increased in positive controls compared to negative controls, but significant changes were not observed in the expression of MMP-2. In addition, treatment of differentiated cells with high concentration of M2000 (25 µg/mL) significantly decreased MMP-2 and MMP-9 mRNA compared to positive controls (Figure 3).

Effect of M2000 on TIMP-1 and TIMP-2 Gene Expressions

Real-time quantitative RT-PCR showed that the expression of TIMP-1 mRNA was markedly higher in positive controls than in negative controls, whereas there were not significant changes in the expression of TIMP-2 mRNA. In addition, treatment of differentiated cells with high concentration of M2000 decreased expression of TIMP-1, but had no significant effect on the expression of TIMP-2 (Figure 4).

Effect of M2000 on MMP-2 and MMP-9 Activities

Gelatinolytic activities of MMP-2 and MMP-9 in the conditioned medium were determined by electrophoresis of the soluble protein in a 10% polyacrylamide gel. The results indicate that PMA is able to increase MMP-9 activity but MMP-2 activity remained unchanged. Furthermore, M2000 significantly inhibited the activities of MMP-2 (63 kDa) in both used concentrations and, of MMP-9 (82 kDa) only at high concentration (25 µg/mL) (Figure 5).

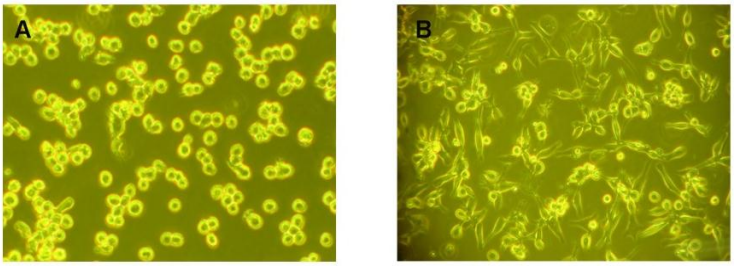

Figure 1. All experiments were performed on the differentiated THP-1 cells. For this purpose, cells were treated by 25 ng/mL PMA for 24 h. A: Microscopic image of cells before differentiation. Round cells were monocyte. B: Microscopic image of cells after differentiation into macrophage. Cells became amoeboid-shape and adhered to cell culture plate.
PMA; phorbol myristate acetate

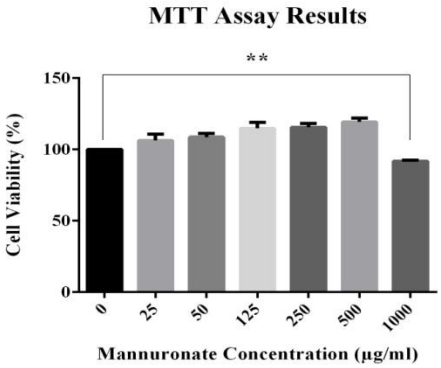

Figure 2. Effect of M2000 on the viability of PMA-differentiated THP-1 cells by MTT assay. Cells were incubated with M2000 concentrations of 0, 25, 50, 125, 250, 500, and 1000 µg/mL in RPMI-1640 medium with 10% FBS. Absorbance was measured at 570 nm. Statistical analysis of results showed no significant changes in cell viability except in concentration of 1000 µg/mL that about 90% of cells survived. Significant deference compared to control: *<0.05, **<0.01, *<0.001, ****<0.0001**
M2000; β-D-Mannuronic Acid, PMA; phorbol myristate acetate, MTT; 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, RPMI; Roswell Park Memorial Institute, FBS; fetal bovine serum

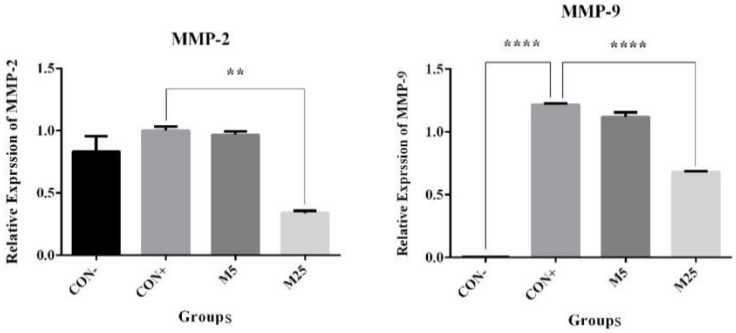

Figure 3. Effect of M2000 on expression of MMP-2 and MMP-9 mRNAs in PMA-differentiated THP-1 cells. The real-time quantitative RT-PCR was carried out and relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ method. The results show the means±S.E.M. of at least six independent experiments. Significant deference compared to positive control: *<0.05, **<0.01, *<0.001, ****<0.0001**
M2000; β-D-Mannuronic Acid, MMP; matrix metalloproteinase, PMA; phorbol myristate acetate, RT-PCR; reverse transcription polymerase chain reaction, CON-; Negative control, CON+; Positive control, M5; 5 µg/mL of Mannuronic acid, M25; 25 µg/mL of Mannuronic acid

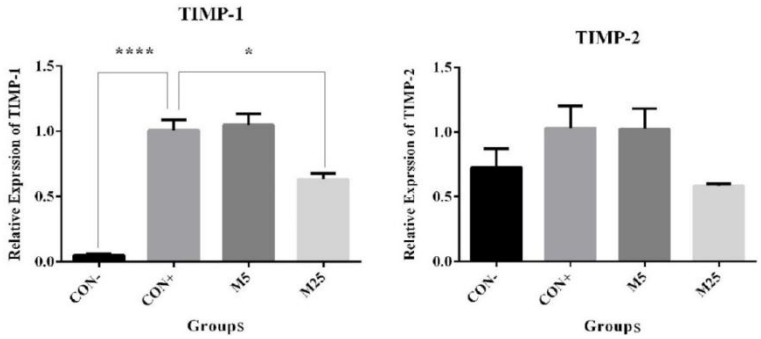

Figure 4. Effect of M2000 on expression of TIMP-1 and TIMP-2 mRNAs in PMA-differentiated THP-1 cells. The real-time quantitative RT-PCR was carried out and relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ method. The results show the means±S.E.M. of at least six independent experiments. Significant deference compared to positive control: *<0.05, **<0.01, *<0.001, ****<0.0001**

M2000; β-D-Mannuronic Acid, TIMP; tissue inhibitor of matrix metalloproteinase, PMA; phorbol myristate acetate, RT-PCR; reverse transcription polymerase chain reaction, CON-; Negative control, CON+; Positive control, M5; 5 µg/mL of Mannuronic acid, M25; 25 µg/mL of Mannuronic acid

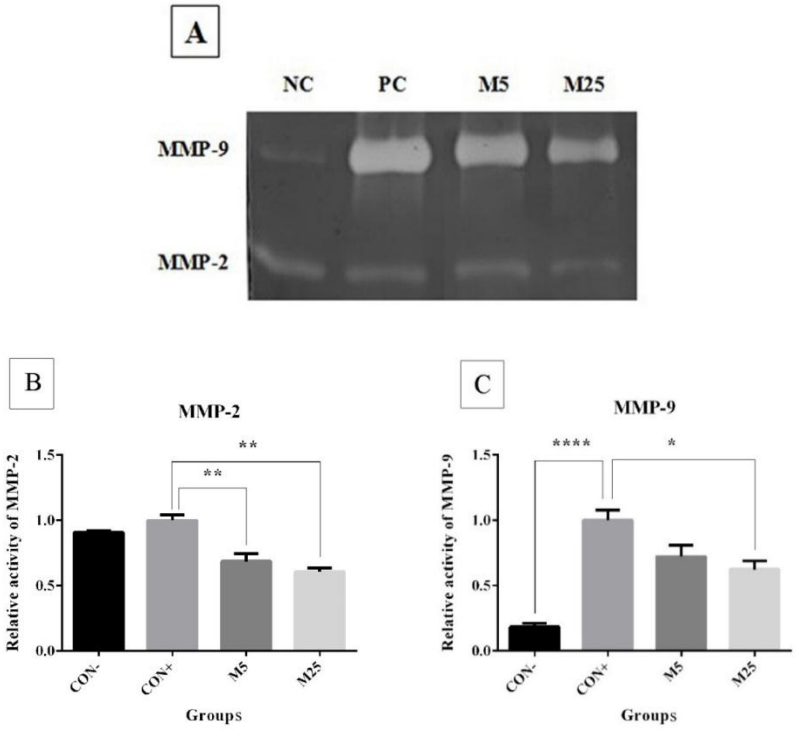

Figure 5. Effect of M2000 on gelatinolytic activities of MMP-2 and MMP-9. After collection of the supernatant and adjustment of their concentration by bradford, gelatinolytic activity of MMP-2 and MMP-9 in the concentrated conditioned medium were assessed by zymography. A: Image of zymography gel. Clear bands have been created as a result of gelatinolytic activity of MMP-2 and MMP-9; B: Densitometric analysis of MMP-2 activity; C: Densitometric analysis of MMP-9 activity. The results represent the means±S.E.M. of at least three independent experiments. Significant deference compared to positive control: *<0.05, **<0.01, *<0.001, ****<0.0001.**

M2000; β-D-Mannuronic Acid, MMP; matrix metalloproteinase, CON-; Negative control, CON+; Positive control, M5; 5 µg/mL of Mannuronic acid, M25; 25 µg/mL of Mannuronic acid

Effect of M2000 on Cell Surface Expression of CD147 in PMA-Differentiated THP-1 Cells

Flow cytometry analysis showed that cell surface expression of CD147 was significantly increased in positive controls compared to negative controls. In addition, treatment of differentiated cells with high concentration of M2000 (25 µg/mL) significantly decreased CD147 expression compared to the positive control (Figure 6).

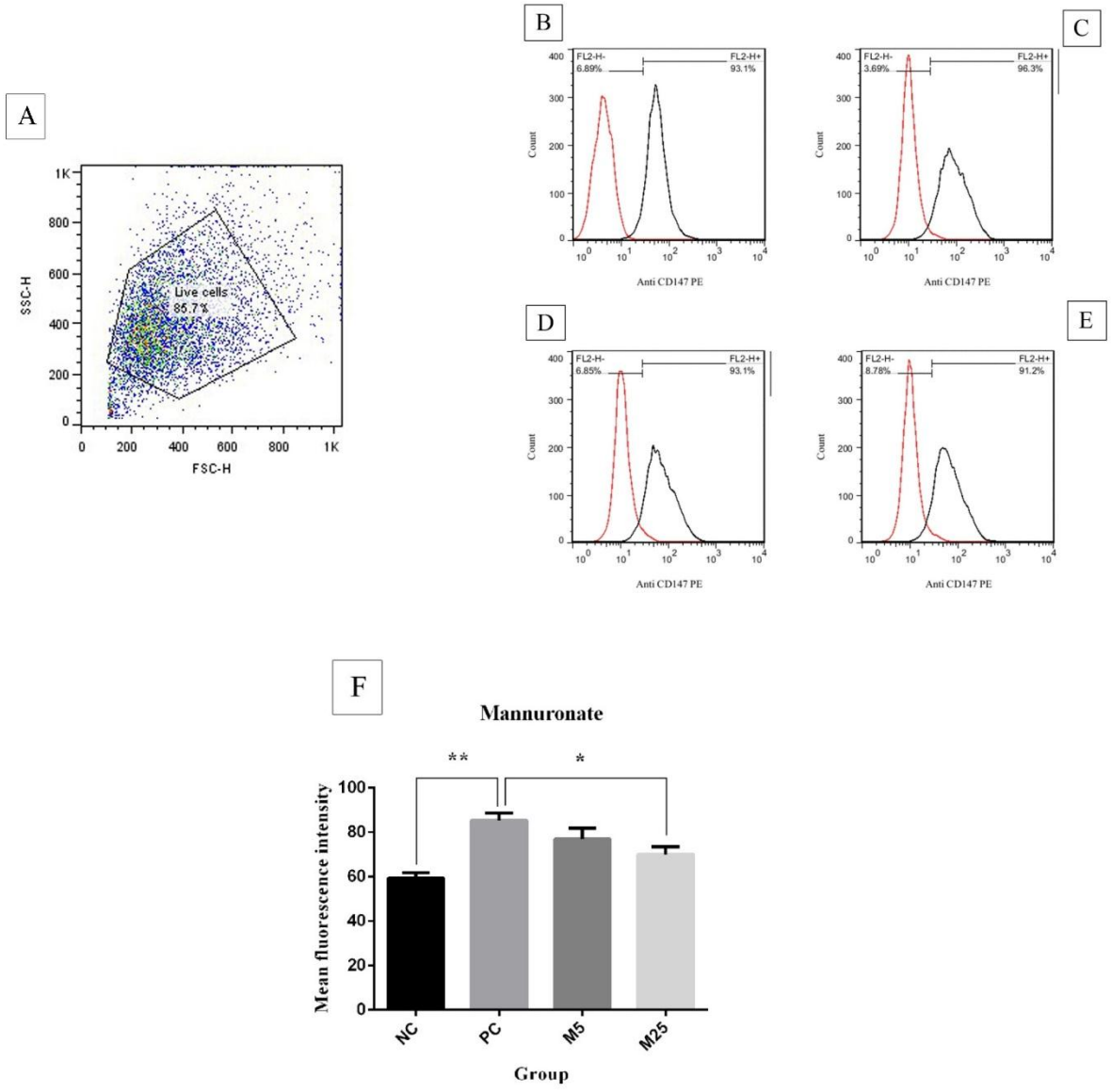

Figure 6. Effect of M2000 on expression of CD147 protein in PMA-differentiated THP-1 cells. Cell surface expression of CD147 was determined after treatment with phycoerythrin-labeled mouse anti-human CD147 antibodies and phycoerythrin-labeled mouse IgG1 antibodies as a control. A: Representative dot plot and live cells gating. B: Negative control; C: Positive control; D: M2000 at 5 µg/mL; E: M2000 at 25 µg/mL; F: Comparison chart of MFI) of groups. The results represent as means±S.E.M. of 3 independent experiments. Significant deference compared to positive control: *<0.05, **<0.01, *<0.001, ****<0.0001.**

M2000; β-D-Mannuronic Acid, PMA; phorbol myristate acetate, MFI; mean fluorescence intensity, NC; Negative control, PC; Positive control, M5; 5 µg/mL of Mannuronic acid, M25; 25 µg/mL of Mannuronic acid

DISCUSSION

M2000 is a novel designed non-steroidal anti-inflammatory drug that its anti-inflammatory and immunosuppressive properties have been demonstrated in experimental models of diseases such as rheumatoid arthritis, multiple sclerosis, glomerulonephritis and nephritis.[20] However, its possible mechanisms are not completely understood.

Previous research indicated that M2000 inhibits MMP-2 activity in WEHI-164 cell line.[21] According to this inhibitory effect and also the role of matrix metalloproteinases, particularly gelatinases and monocyte/macrophage cells in the development of inflammatory diseases; we studied the effects of M2000 on MMP-2 and MMP-9 as a possible anti-inflammatory mechanism in differentiated THP-1 cell line.

THP-1 cell line is derived from human myeloid leukemia that can be differentiated into macrophage by PMA. It is a common model for the study in the field of monocytes and macrophages which is widely used to investigate the effect of various compounds.[22]

Previous cytotoxicity analysis of M2000 compared with diclofenac, piroxicam, and dexamethasone in the fibrosarcoma cell line showed that M2000 had a much higher tolerability compared with other tested drugs.[23] In order to confirm the safety of the drug in the THP-1 cell line, these cells were treated with various concentrations of M2000 (5, 25, 50,125,250,500, and 1000 μg/mL). Our results showed that there was no significant difference at all concentrations, except at the concentration of 1000 μg/mL that reduced cell viability by about 10%.

THP-1 cells are differentiated to macrophages by PMA and became flat and amoeboid in shape, and adhered to the bottom of cell culture plates.[24] Followed by differentiation, surface expression of CD147, activity and expression of MMP-2 and MMP-9 mRNA are increased.[25] Our results also showed that surface expression of CD147 increases in cells treated with PMA. In contrast, a high concentration of M2000 inhibits this increased expression.

Gelatinases expression and activity, particularly MMP-9 increase, have been associated with increased expression of CD147 during differentiation of THP-1 cells,[25] which was also confirmed by our data . In contrast, high concentration of M2000 reduces gene expression and activity of gelatinases and probably this effect is applied through CD-147.

Tissue inhibitors of matrix metalloproteinases play an important role in balancing ECM through inhibition of MMPs activity, so that the increase and decrease of TIMPs levels result in ECM accumulation and proteolysis, respectively.[26] TIMPs are classified into four groups, including TIMP-1, 2, 3, and 4. TIMPs are able to inhibit the activity of a wide range of MMPs,[2] whereas the role of TIMP1 and TIMP-2 is to inhibit the MMP-9 and -2 activities, respectively.[14] The imbalance between TIMP-1/TIMP-2 and MMP-9/MMP-2 has been seen in various diseases. In a study, it was shown that PMA increased the gene expression of TIMP- 1, but had no significant impact on the expression of TIMP-2 [24], since the increased MMP-9 gene expression and activity resulted to a reduced expression in TIMP-1. This fact is in agreement with our findings in connection with the reduction of TIMP-1 gene expression following the treatment of cells with M2000.

The limitation of this study was inaccessibility to appropriate animal model to confirm the results of in vitro experiments

In summary, our findings show that M2000 is able to decrease gene expression and activity of MMP-2 and MMP-9 by inhibiting CD147 in PMA-differentiated THP-1 cells. In addition, increased MMP-9 gene expression and activity results in reduced TIMP-1 expression in these cells.

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