Tropical Journal of Pharmaceutical Research August 2015; 14 (8): 1357-1364
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v14i8.6

Original Research Article

Pinitol Suppresses Tumor Necrosis Factor-α-Induced Invasion of Prostate Cancer LNCaP Cells by Inhibiting Nuclear Factor-κB-Mediated Matrix Metalloproteinase-9 Expression

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Received: 4 May 2015 *Revised accepted: 10 June 2015*

Abstract

Purpose: *To investigate the mechanism by which pinitol inhibits tumor necrosis factor-α (TNF-α)-induced expression of matrix metalloproteinase-9 (MMP-9) and invasion of prostate cancer LNCaP cells.*
Methods: *Reverse transcription-polymerase chain reaction (RT-PCR) together with Western blot analysis was used to analyze the expression of MMP-9 and nuclear factor-κB (NF-κB) subunits, p65 and p50, in TNF-α-treated LNCaP cells, while 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, flow cytometry, and DNA fragmentation were used to evaluate cell viability and apoptosis. MMP-9 activity and invasion were measured by gelatin zymography and matrigel invasion assay, respectively. DNA-binding activity of NF-κB and AP-1 was determined by electrophoretic mobility shift assay and luciferase activity.*
Results: *MMP-9 activity significantly increased in response to TNF-α; however, pinitol reduced TNF-α-induced MMP-9 activity without cytotoxicity. Matrigel invasion assay showed that pinitol reduced TNF-α-induced invasion of prostate cancer LNCaP cells. Further, it downregulated the expression of MMP-9 gene induced by TNF-α-treatment. Pinitol suppressed TNF-α-induced NF-κB activity by suppressing nuclear translocation of the NF-κB subunits, p65 and p50.*
Conclusion: *The results indicate that pinitol is a potential anti-invasive agent and acts by suppressing TNF-α-induced cancer cell invasion and specifically inhibiting NF-κB as well as downstream target genes such as MMP-9.*

Keywords: *Pinitol, Matrix metalloproteinase-9, Cell invasion, Nuclear factor-κB, Nuclear translocation*

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading all components of the extracellular matrix. Degradation of the basement membrane and extracellular matrix by MMPs facilitates the invasion of malignant cells through connective tissues and blood vessel walls and results in the establishment of metastases [1]. Most MMPs are secreted as inactive zymogens and are extracellularly activated, where their functions are tightly regulated by several different mechanisms [2,3].

MMP-9 contributes to invasion and metastasis, and is thought to be a key enzyme in the degradation of type IV collagen, which is a major component of the basement membrane [4] and is abundantly expressed in various malignant tumors including prostate, bladder, brain, liver, and pancreatic carcinoma [5]. Clinical investigations reveal that prostate cancer can metastasize to distant organs including the liver, bladder, bone, lungs, spine, and lymph nodes [6,7]. Therefore, a good strategy to treat prostate cancers is to target MMP-9 expression and activity.

The transcription factor nuclear factor kappa-B (NF-κB) is involved in the regulation of several invasion-related genes such as MMP-9 in cancer cells [8]. NF-κB can act directly by binding to the MMP-9 promoter or indirectly via activation of other transcription factors. It has recently been reported that the inhibition of NF-κB activity decreases the tumorigenic and metastatic ability of human prostate cancer cells by suppressing angiogenesis and invasion through the downregulation of MMP-9 [9,10]. Therefore, targeting the NF-κB pathway is considered as a potential strategy to suppress MMP-9-mediated tumor invasion.

Pinitol, traditionally used in Ayurvedic medicine and extracted from plants such as *Bougainvillea spectabilis*, has been shown to exhibit anti-inflammatory and antidiabetic activities, inhibit the T-helper cell-1 response, and prevent cardiovascular diseases [11-13]. Pinitol appears to regulate the effects of insulin by targeting downstream in the insulin signaling pathway, suggesting that pinitol possesses hypoglycemic activity [14]. Pinitol also triggers apoptotic cascades in human breast cancer cells by inducing the expression of tumor suppressor genes [12]. However, little is known about the effects of pinitol on the expression and invasion of MMP-9 in prostate cancer cells.

In this study, the effects of pinitol on MMP-9 expression and invasion in TNF-α-stimulated prostate cancer LNCaP cells were evaluated. It was found that pinitol downregulated TNF-α-induced MMP-9 expression and invasion by suppressing NF-κB activation.

EXPERIMENTAL

Reagent and antibodies

Pinitol was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO (vehicle). Antibodies against MMP-9, p65, p50, β-actin, and C23 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MMP-9 inhibitor I (MMP-9-I) was obtained from Merck (Darmstadt, Germany). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) and propidium iodine (PI) were obtained from Sigma Chemical Co.

Cell culture and viability

Human prostate cancer LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI medium (WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% heat-inactivated FBS (WelGENE Inc.) and 1 % penicillin-streptomycin (WelGENE Inc.) in 5 % CO_2 at 37 °C. Cells were seeded at 1 × 10^5 cells/ml and then treated with various concentrations of pinitol. After 24-h incubation, the viability was determined by an MTT assay.

Flow cytometric analysis

Apoptotic sub-G1 phase was analyzed by PI-stained cells. Briefly, LNCaP cells (1 × 10^6) were fixed in 70 % ethanol overnight at 4 °C. The cells were washed in phosphate-buffered saline (PBS) with 0.1 % BSA and incubated with 1 U/ml of RNase A (DNase free, Sigma) and 10 µg/ml of PI overnight at room temperature in the dark. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA). The levels of apoptotic cells with sub-G1 phase were determined as a percentage of the total number of cells.

DNA fragmentation assay

LNCaP cells were treated with the indicated chemicals and then lysed on ice in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5 % Triton X-100 for 30 min. Lysates were vortexed and cleared by centrifugation at 10,000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and electrophoretically analyzed on a 1.5 % agarose gel containing ethidium bromide.

Wound healing assay

LNCaP cells were grown to 90 % confluence in a 6-well plate in 5 % CO_2 at 37 °C. A wound was created by scratching cells with a sterile 200 µl pipette tip. The cells were washed twice with PBS to remove floating cells and then added to a medium without serum for 24 h. Images of the

wound were taken under × 100 magnitude microscope.

Gelatin zymography

Cultured LNCaP cells were harvested and washed with serum-free RPMI medium three times, and incubated for 24 h at 5 × 10^5 cells/ml in serum-free RPMI medium (conditioned medium). The cells were administrated with pinitol in the presence or absence of TNF-α. MMP-9 activity was determined by gelatin zymography using 0.1 % gelatin as a substrate. The conditioned medium was mixed with SDS-PAGE sample buffer in the absence of reducing agent and electrophoresed in 8 % polyacrylamide gel. After electrophoresis, the gels were washed three times with 2.5 % Triton X-100 in water and then incubated overnight in a closed container at 37 °C in 0.2 % Brij 35.5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris (pH 7.4). The gels were stained for 30 min with 0.25 % Coomassie Blue in 10 % acetic acid and 45 % methanol, and then destained for 30 min using an aqueous mixture with 20 % acetic acid, 20 % methanol, and 17 % ethanol. Areas of protease activity appeared as clear bands.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Amersham, Arlington Heights, IL). The detection of specific proteins was carried out with an ECL Western blotting kit (Amersham) according to the recommended procedure.

RNA extraction and RT-PCR

Total RNA was isolated using Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA that was reverse-transcribed from 1 µg of total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology). Primers for MMP-9 sense (5′-CCT GGA GAC CTG AGA ACC AAT CT-3′); MMP-9 antisense (5′-CCA CCC GAG TGT AAC CAT AGC-3′); and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) sense (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′); GAPDH antisense (5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) were used. PCR reaction was initiated at 94 ºC for 2 min followed by 31 cycles of 94 ºC for 30 min, 30-min annealing temperature, 72 ºC for 30 min followed by final extension at 72 ºC for 5 min. Annealing temperatures for MMP-9 and GAPDH were 63 ºC and 62 ºC, respectively. After amplification, PCR products were separated on 1.5 % agarose gels and visualized by ethidium bromide fluorescence.

Invasion assay

Invasion assays were performed using modified Boyden chambers with polycarbonate nucleopore membrane (Corning, Corning, NY). Precoated filters (6.5 mm in diameter, 8 µm pore-size, Matrigel 100 µg/cm2) were rehydrated and 5 × 10^4 cells in medium with or without pinitol or MMP-9 inhibitor I (5 nM) in the presence or absence of TNF-α (20 ng/ml) were seeded into the upper part of each chamber. After 24-h incubation, nonmigratory cells on the upper surface of the filter were wiped with a cotton swab and migrated cells on the lower surface of the filter were fixed and stained with 0.125 % Commassie Blue in a methanol:acetic acid:water mixture (45:10:45, v/v/v). Random fields were counted under a light microscope.

Luciferase assay

NF-κB and AP-1 reporter construct were purchased from Clontech (Palo Alto, CA). Briefly, LNCaP cells were plated onto six-well plates at a density of 5 × 10^5 cells/well and grown overnight. Cells were transfected with 2 µg of each plasmid construct for 6 h by the Lipofectamine method. After transfection, the cells were cultured in 10 % FBS containing RPMI medium with various concentrations of pinitol in the presence of 20 ng/ml TNF-α for 24 h. Cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.8, 1 % Triton X-100, 150 mM NaCl, and 2 mM DTT). Five µl of cell lysate was mixed with luciferase 25 µl activity assay reagent and luminescence produced for 5 s was measured using GLOMAX luminometer (Promega, Madison, WI).

Electrophoretic mobility shift assay (EMSA)

The preparation of cytoplasmic and nuclear extracts was conducted using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB-binding oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) (Santa Cruz Biotechnology) were biotinylated using the biotin 30-end DNA labeling kit (Pierce) according to the manufacturer's instructions and annealed for 1 h at room temperature. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/ml poly(dI-dC), 0.05 % Nonidet P-40, 5 mM $MgCl_2$, 10 mM EDTA, and

2.5 % glycerol in 1× binding buffer (LightShift™ chemiluminescent EMSA kit) with 20 fmol of biotin-end-labeled target DNA and 10 µg of nuclear extract. Assays were loaded onto native 4 % polyacrylamide gels pre-electrophoresed for 60 min in 0.5x Tris borate/EDTA and before being transferred onto a positively charged nylon membrane (Hybond™-N$^+$) in 0.5x Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer's instructions.

Statistical analysis

The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Adobe Photoshop (version 8.0). All data are presented as mean ± SE. Significant differences between the groups were determined using one-way ANOVA test. $P < 0.05$ was adopted as an indication of statistical significance.

RESULTS

Pinitol has no effect on LNCaP cell viability with inhibition of migration

In order to determine the cytotoxic potential of pinitol in LNCaP cells, an MTT assay was performed 24 h after treatment with the indicated concentrations of pinitol in the presence of FBS. Pinitol with and without TNF-α was not cytotoxic at any of the concentrations tested in the study (Fig 1A). DNA fragmentation was analyzed and it was observed that neither pinitol nor pinitol with TNF-α-treated groups showed fragmented DNA compared with the positive H_2O_2-treated group (Fig 1B). Therefore, pinitol levels below 400 µM were selected for subsequent experiments.

The effects of pinitol on cell viability and cytotoxicity were analyzed in detail. According to the percentages of sub-G1 DNA contents by flow cytometry, there was no apoptotic cell death in either panel compared to the positive H_2O_2-treated group (Fig 1C). Additionally, to determine whether pinitol inhibits TNF-α-induced migration, a scratch wound healing assay was carried out. Cell migration was significantly increased by TNF-α treatment and the effects were inhibited in the presence of pinitol (Fig 1D). Taken together, these data indicate that pinitol inhibits TNF-α-induced migration, and that the acquisition of migration is related to tumor invasion without cytotoxicity.

Pinitol suppresses TNF-α-induced MMP-9 expression and invasion of LNCaP cells

Zymography, RT-PCR, and a western blot analysis were conducted to assess whether pinitol regulates MMP-9 expression and activity. The zymography data showed that treatment with TNF-α significantly increased gelatin degradation of MMP-9 in LNCaP cells; however, pinitol suppressed TNF-α-induced MMP-9 activity (Fig 2A). Further, because zymography was performed under serum-free conditions, an MTT assay was performed 24 h after treatment with the indicated concentrations of pinitol in the absence of FBS.

Pinitol with or without TNF-α was not cytotoxic at any of the concentrations tested in this study (Fig 2B). It was also found that pinitol downregulated TNF-α-induced MMP-9 mRNA and protein expression. In the western blot analysis, TNF-α stimulation of the cells resulted in a significant increase in MMP-9 expression compared to that in the untreated control; however, pinitol reduced TNF-α-induced MMP-9 expression to the levels of the untreated control (Fig 2C). Moreover, MMP-9 gene expression was confirmed by RT-PCR analysis. Pretreatment with pinitol significantly suppressed TNF-α-induced MMP-9 upregulation at the mRNA level (Fig 2D). Taken together, these results indicate that pinitol suppresses TNF-α-stimulated MMP-9 expression at the transcriptional level.

MMP-9 is thought to be significantly involved in the processes of tumor invasion and angiogenesis. Since pinitol inhibited MMP-9 expression and activity, the effects of pinitol on invasion of LNCaP cells were examined. When the cells were treated with TNF-α alone, a remarkable 4-fold increase in cell invasion was observed compared to that in the untreated control (Fig 2E). However, pinitol pretreatment resulted in 50 % reduction in TNF-α-induced penetration through a matrigel-coated membrane compared to that in the TNF-α-treated group (Fig 2E). These results confirm that pinitol inhibits TNF-α-induced invasion of prostate cancer LNCaP cells.

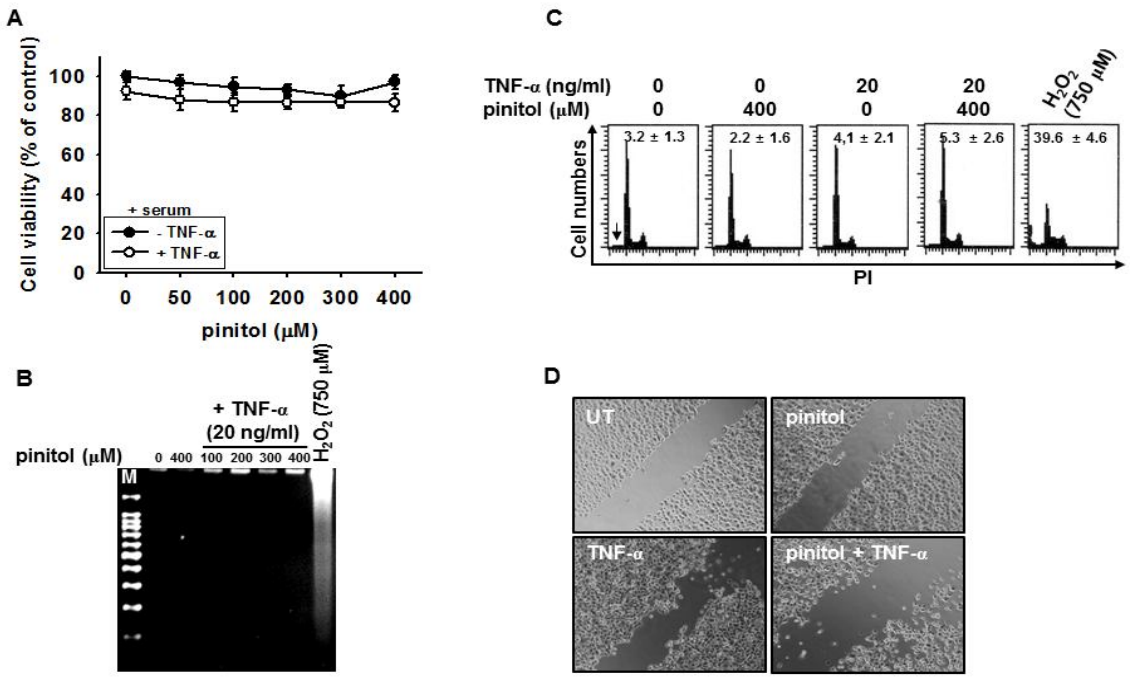

Fig 1: Effect of pinitol on the viability of LNCaP cells. LNCaP cells were treated with the indicated concentrations (0 - 400 μM) of pinitol and/or 20 ng/ml TNF-α in the presence of FBS for 24 h. (A) Cell viability was measured by an MTT assay. (B) Total DNA was extracted from the treated cells and the DNA fragmentation assay was analyzed on a 1.5 % agarose gel. (C) DNA content was analyzed by flow cytometry. The percentage of sub-G_1 DNA content is indicated in each panel. The data and arrow in the figure indicate the percentage of each sub-G_1 phase in the cell cycle distribution. (D) For the wound healing assay, cells were treated with pinitol in the presence or absence of 20 ng/ml TNF-α in a six-well plate and incubated for 24 h. Images of the wound were taken under a microscope at ×100 magnitude

Pinitol downregulates TNF-α-induced NF-κB activity, not AP-1

In order to determine whether MMP-9 mRNA expression was regulated by NF-κB or AP-1 activity, a promoter assay was carried out using transiently transfected LNCaP cells with a luciferase reporter vector that included the NF-κB or AP-1 binding sites. NF-κB luciferase activity increased approximately 10-fold in TNF-α-treated LNCaP cells compared to that in the untreated control group (Fig 3A). TNF-α-stimulated luciferase activity in the cells containing the NF-κB construct was significantly reduced by treatment with pinitol. However, no significant change in AP-1 luciferase level was observed in response to pinitol or TNF-α, suggesting that pinitol regulates TNF-α-induced MMP-9 expression independently of AP-1.

The specific DNA-binding activity of NF-κB was assessed by EMSA to investigate whether pinitol inhibited MMP-9 activity by suppressing NF-κB. TNF-α-stimulation caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA at 30 min; pretreatment with pinitol significantly reduced TNF-α-induced NF-κB activity (Fig 3B).

In a similar experiment, expression levels of p65 and p50 in the cytoplasmic region were determined after TNF-α-stimulation. TNF-α significantly lowered p50 and p65 expression in the cytoplasmic compartments of LNCaP cells; however, treatment with pinitol sustained the TNF-α-induced expression of p50 and p65 (Fig 3C). Treatment with pinitol also attenuated TNF-α-induced nuclear translocation of p50 and p65. These results indicate that pinitol inhibits MMP-9 expression by suppressing the NF-κB signaling pathway.

DISCUSSION

Based tumor invasion and metastasis are multistep processes by which a subset of individual cancer cells disseminate from a primary tumor to distant secondary organs or tissues. MMPs play a major role in promoting tumor invasion and metastasis; MMP-9, in particular, is regarded as a critical molecule in regulating the progression of tumor invasion and metastasis [15]. Thus, the inhibition of MMP-9

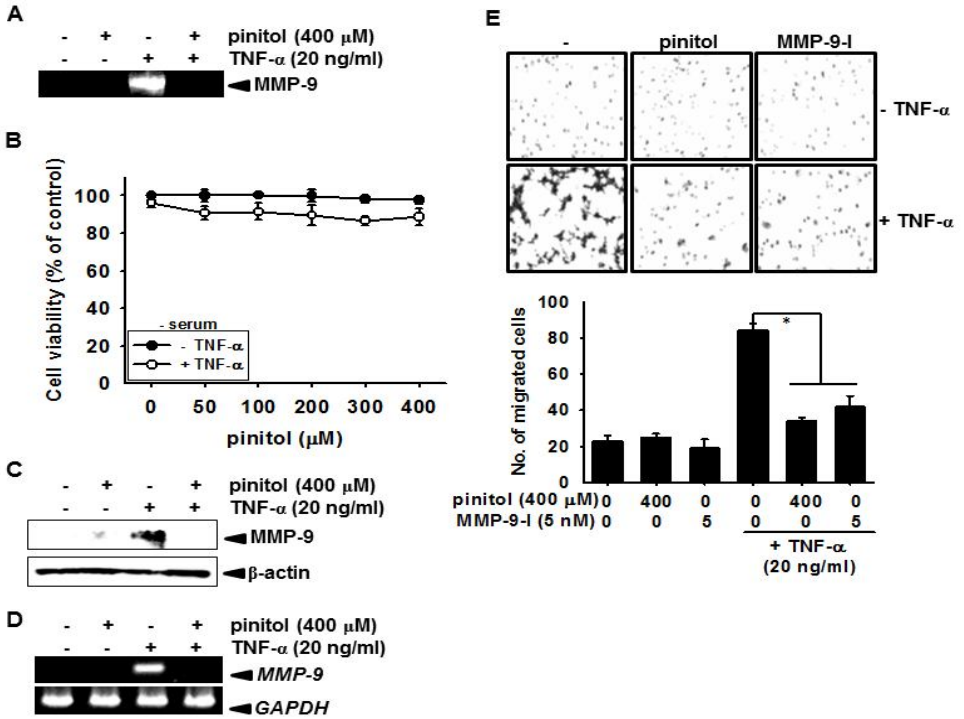

Fig 2: Effects of pinitol on TNF-α-induced MMP-9 expression and invasion of LNCaP cells. (A) LNCaP cells were treated with 400 μM pinitol 1 h before treatment with 20 ng/ml TNF-α in the absence of FBS for 24 h. Conditioned medium was collected, and subjected to gelatin zymography. (B) Cell viability was measured in the absence of FBS by an MTT assay at 24 h. (C) Equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against MMP-9. (D) LNCaP cells were incubated with the indicated concentrations of pinitol 1 h before treatment with 20 ng/ml TNF-α for 6 h. Total RNA was isolated, and RT-PCR analysis of MMP-9 was performed. (E) The upper part of the transwells was coated with matrigel for the invasion assay. The cells were then cultured in serum-free media for 3 h before treatment with 400 μM pinitol in the absence or presence of 20 ng/ml TNF-α. After 24-h incubation, the cells passing through the matrigel to the membrane were dyed using 0.125 % Coomassie Blue in ethanol. Statistical significance was determined by a one-way ANOVA test (*, $p < 0.05$ *vs*. TNF-α-treated group). ***Note:*** GAPDH and β-actin were used as internal controls for the RT-PCR and western blot analysis, respectively. MMP-9-I = MMP-9 inhibitor

expression is an important therapeutic experimental model of tumor invasion. The results demonstrate that pretreatment with pinitol inhibits TNF-α-induced MMP-9 activity accompanied by suppression of MMP-9 gene transcription in prostate cancer LNCaP cells. Additionally, the matrigel assay shows that pinitol suppresses TNF-α-induced invasion of LNCaP cells.

Although NF-κB plays a key role in regulating the immune response to infection, aberrant or incorrect regulation of NF-κB has been linked to improper inflammatory immune responses. Several studies have also identified the signal transduction pathways involved in regulating MMP-9 expression in various cells [16,17]. In particular, NF-κB is an important transcription factor for regulating the MMP-9 gene expression containing NF-κB binding sites [8]. In response to stimuli, IκB is phosphorylated and degraded, and NF-κB is released and subsequently translocated to the nucleus [18].

Since expression of the MMP-9 gene is regulated at the transcriptional level by the NF-κB/Rel family of transcription factors [17], the expression of p65 and p50 was tested, as well as the specific DNA-binding activity of NF-κB. In this study, it was shown that pinitol regulates the NF-κB activity by inhibiting p65 and p50 protein translocation. The results suggest that the downregulation of NF-κB by pinitol could potentiate anti-invasive activities via the downregulation of MMP-9 expression. Further, it was shown that the TNF-α-stimulated luciferase activities in LNCaP cells were significantly reduced in response to pinitol.

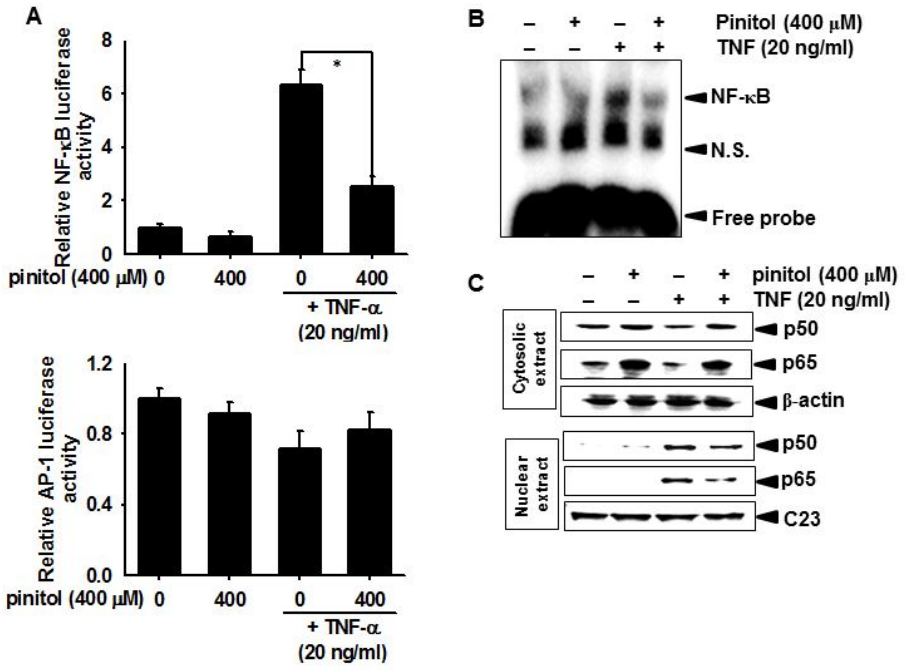

Fig 3: Effects of pinitol on NF-κB-DNA binding activity. LNCaP cells were treated with 400 μM pinitol in the presence of 20 ng/ml TNF-α. (A) Cells were transfected with WT-NF-κB and AP-1 promoter-containing reporter vectors, and luciferase activity was measured 24 h after transfection. Cells were preincubated with 400 μM pinitol 1 h before treatment with 20 ng/ml TNF-α for 9 h. (B) Nuclear extracts were assayed for NF-κB activity by EMSA at 30 min and (C) the levels of p50 and p65 were analyzed by western blot analysis. Statistical significance was determined by a one-way ANOVA test (*, $p < 0.05$ *vs*. TNF-α-treated group). ***Note:*** β-Actin and C23 were used as an internal control for the western blot analysis

It has been reported that several transcription factors in the human MMP-9 promoter region such as AP-1 and SP1, also regulate MMP-9 expression in response to lipopolysaccharide, phorbol 12-myristate 13-acetate (PMA), and TNF-α [19,20]. The AP-1 site at approximately −70 bp upstream of the transcriptional start site has long been thought to play a dominant role in the transcriptional activation of the MMP promoters in response to PMA [21]; however, pinitol could not regulate TNF-α-stimulated AP-1 activity in this study. The role of SP1 in regulating expression of MMP-9 has not been determined in this study, although SP1 sites of the MMP-9 promoter are important transcriptional elements [22]. Therefore, further experiments are needed to confirm the role of SP1 in regulation of TNF-α-induced MMP-9 by pinitol.

CONCLUSION

Findings from this study suggest that treatment with pinitol plays an important role in the regulation of MMP-9, resulting in the inhibition of invasion of prostate cancer LNCaP cells. The results also show that pinitol is a potent inhibitor of TNF-α-induced MMP-9 expression and invasion by suppression of the NF-κB pathway.

ACKNOWLEDGEMENT

This research was supported by 2015 Scientific Promotion Program funded by Jeju National University.

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