Abstract. Piceatannol has potent anti-inflammatory, immunomodulatory, anticancer and antiproliferative effects. However, little is known about the mechanism by which piceatannol inhibits invasion and metastasis. The aim of the current study was to investigate the effects of piceatannol on the expression of matrix metalloproteinase-9 (MMP-9) in DU145 human prostate cancer cells. The results revealed that MMP-9 activity was significantly increased in response to tumor necrosis factor-α (TNF-α). However, treatment with piceatannol reversed TNF-α- and MMP-9-induced gelatin zymography and its gene expression. In addition, a Matrigel invasion assay determined that piceatannol reduces the TNF-α-induced invasion of DU145 cells. Nuclear factor-κB (NF-κB) is a significant transcription factor that regulates numerous genes involved in tumor cell invasion and metastasis. Therefore, whether piceatannol acts on NF-κB to regulate MMP-9 gene expression was analyzed. The results revealed that piceatannol attenuates MMP-9 gene expression via the suppression of NF-κB activity. Using a specific NF-κB inhibitor, pyrrolidine dithiocarbamate, it was confirmed that TNF-α-induced MMP-9 gene expression is primarily regulated by NF-κB activation. Piceatannol inhibited NF-κB activity by suppressing nuclear translocation of the NF-κB p65 and p50 subunits. Furthermore, TNF-α-induced Akt phosphorylation was significantly downregulated in the presence of piceatannol. The Akt inhibitor LY294002 caused a significant decrease in TNF-α-induced NF-κB activity and MMP-9 gene expression. Overall, these data suggest that piceatannol inhibits TNF-α-induced invasion by suppression of MMP-9 activation via the Akt-mediated NF-κB pathway in DU145 prostate cancer cells.

Introduction

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, are involved in the degradation of the extracellular matrix (ECM) under normal physiological conditions and during the metastatic process (1). While MMP activity is normally tightly regulated, dysregulation of MMP activity has been linked to cancer progression and metastasis via the degradation of the basement membrane and the induction of angiogenesis (2). The degree of overexpression of certain MMPs has been noted to correlate with the stage of disease and/or prognosis (3). The majority of MMPs are secreted as inactive zymogens that are activated extracellularly and their functions are tightly regulated by several mechanisms (4). MMP-9 is a key effector molecule that promotes tumor cell invasion through type-IV collagen degradation-dependent extracellular matrix remodeling (5). MMP-9 expression has been observed in tumors of various organs, including the prostate, bladder, brain, liver and pancreas (6). The growth and metastasis of prostate cancer, as well as other tumors, is dependent on the formation
of new blood vessels from preexisting ones via angiogenesis. Growing evidence suggests that prostate cancer cells secrete high levels of growth factors and matrix-degrading proteases, thus allowing metastasis to distant organs, including the liver, lungs, spine, bladder, bone and lymph nodes (7). Therefore, targeting MMP-9 inhibition for treating prostate cancers is considered to be an effective strategy.

A previous study has shown that the nuclear factor κB (NF-κB) pathway tightly regulates MMP-9 expression in several types of cancer cells (8). NF-κB is normally located in the cytoplasm as an inactive dimer. The activity of NF-κB is regulated by interaction with inhibitory IκB proteins, which repress the potential of NF-κB to translocate to the nucleus and bind with DNA (9). Upon activation, IκB is phosphorylated, which marks the inhibitor for ubiquitination and degradation via a proteasome-dependent pathway (10). A previous study demonstrated that the inhibition of NF-κB activity in human prostate cancer cells decreases their tumorigenic and metastatic abilities by suppressing angiogenesis and invasion via the downregulation of MMP-9 (11). Conversely, TNF-α stimulation results in NF-κB activation in prostate cancer cells and induces tumor invasion (12). Therefore, a potential strategy to suppress MMP-9-mediated tumor invasion is via the NF-κB pathway.

Piceatannol (3,5,3',4'-tetrahydroxytrans-stilbene), a phenolic compound and an analog of resveratrol, which naturally occurs in grapes and red wine, has been shown to possess anticancer properties via reduction in the expression of the anti-apoptotic protein Bcl-2 and inhibitors of apoptosis (IAP) family, in addition to anti-inflammatory activity via the downregulation of NF-κB (13-15). In a previous study, Kang et al reported the mechanism of piceatannol in terms of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (16). However, the effects of piceatannol on MMP-9 gene expression and invasion in cancer cells have not been evaluated.

The current study examined the effects of piceatannol on MMP-9 expression and invasion in TNF-α-stimulated DU145 prostate cancer cells. It was demonstrated that piceatannol downregulates TNF-α-induced MMP-9 mRNA and protein expression by suppressing NF-κB activation. Furthermore, the regulation of NF-κB activity by piceatannol is associated with the inhibition of the phosphorylation of Akt.

Materials and methods

Reagent and antibodies. Piceatannol was purchased from Tocris (St. Louis, MO, USA) and dissolved in DMSO (vehicle). Antibodies against p65, p50, β-actin, phospho-(p)-Akt and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A specific NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) and a specific Akt inhibitor LY294002 were purchased from Calbiochem (San Diego, CA, USA). MMP-9 inhibitor I was obtained from Merck (Darmstadt, Germany). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodine (PI) were obtained from Sigma (St. Louis, MO, USA).

Cell culture and viability. Human prostate cancer DU145 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (WelGENE, Inc., Daegu, Korea) supplemented with 10% heat-inactivated FBS (WelGENE, Inc.) and 1% penicillin-streptomycin (WelGENE, Inc.) in 5% CO2 at 37°C. Cells were seeded at 1x10⁶ cells/ml and treated with the indicated concentrations of piceatannol. Following a 24-h incubation, the viability was determined by an MTT assay.

Flow cytometric analysis. Cell cycle distribution was analyzed by PI-stained cells. Briefly, cells (1x10⁶) were fixed in 70% ethanol overnight at 4°C. The cells were washed in phosphate-buffered saline (PBS) with 0.1% BSA. Cells were incubated with 1 U/ml RNase A (DNase free, Sigma) and 10 µg/ml PI overnight at room temperature in the dark. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickenson, San Jose, CA, USA). The levels of apoptotic cells with sub-G1 DNA were determined as a percentage of the total number of cells.

DNA fragmentation assay. Cells were treated with the indicated chemicals and then lysed on ice in a 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 x g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and analyzed electrophoretically on a 1.5% agarose gel containing ethidium bromide.

Wound-healing assay. DU145 cells were grown to 90% confluence in a 6-well plate at 37°C in a 5% CO2 incubator. A wound was created by scratching cells with a sterile 200-µl pipette tip, cells were washed twice with PBS to remove floating cells and then added to a medium without serum. Images of the wound were captured under an x100 magnification microscope.

Gelatin zymography. Cultured DU145 cells were harvested and washed with serum-free DMEM three times and incubated for 24 h at 5x10⁶ cells/ml of serum-free DMEM. The cells were stimulated with piceatannol in the presence of TNF-α (20 ng/ml). 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. Following 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 CaCl2, 1 mM NaCl and 50 mM Tris, pH 7.4. The gels were stained for 30 min with 0.25% Coomassie Blue R-250 in 10% acetic acid and 45% methanol and then destained for 30 min using an aqueous mix of 20% acetic acid, 20% methanol and 17% ethanol. Areas of protease activity appeared as clear bands.

RNA extraction and RT-PCR. Total RNA was isolated using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD, USA) 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, Sungnam, Korea). Primers for...
MMP-9 sense (5'-CCTGGAGACCTGAGACCAATCT-3') and antisense (5'-CCACCCGAGTGTACCACTAGC-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense (5'-CCACCATGGCAAATTCCATGGCA-3') and antisense (5'-TCTAGACGGCAGTCAAGTCTCAC-3') were used. The PCR was initiated at 94°C for 2 min followed by 31 cycles of 94°C for 30 min, 30 min annealing temperature, 72°C for 30 min followed by final extension at 72°C for 5 min. The annealing temperatures for MMP-9 and GAPDH were 63°C and 62°C, respectively. Following amplification, PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence.

**Invasion assay.** Cells (5x10^4/chamber) were used for each invasion assay. Invasion assays were performed using modified Boyden chambers with polycarbonate nucleopore membrane (Corning, Corning, NY, USA). Precoated filters (6.5 mm in diameter, 8 µm pore-size, Matrigel 100 µg/cm²) were rehydrated and 5x10^4 cells in medium with or without piceatannol or MMP-9 inhibitor I (5 nm) in the presence of TNF-α were seeded into the upper part of each chamber. Following 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% Commissie 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 reporter construct was purchased from Clonetech (Palo Alto, CA, USA) and MMP-9 reporter was obtained from Professor Y.H. Choi (Oriental Medicine, Dongeui University, Busan, Korea). Briefly, DU145 cells were plated onto six-well plates at a density of 5x10^5 cells/well and grown overnight. Cells were transfected with 2 µg of each plasmid construct for 6 h by the Lipofectamine method. Following transfection, the cells were cultured in 10% FBS containing DMEM with the indicated concentrations of piceatannol 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). The cell lysate (5 µl) was mixed with luciferase activity assay reagent (25 µl) and luminescence produced for 5 sec was measured using GLOMAX luminometer (Promega, Madison, WI, USA).

**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, USA). DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB-binding oligonucleotides (5'-AGTTAGGGGACTTTCCCAGGC-30', 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 1X 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% poly-
araylaldehydes pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA and transferred onto a positively charged nylon membrane (HybondTM-N+) in 0.5X Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm² and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer's instructions.

**Western blot analysis.** Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). The detection of specific proteins was carried out with an ECL western blotting kit (Amersham) according to the manufacturer's instructions.

**Statistical analysis.** All data were derived from at least three independent experiments. 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 considered to indicate a statistically significant difference.

**Results**

**Piceatannol does not affect DU145 cell viability.** In order to determine the cytotoxic potential of piceatannol on DU145 cells, an MTT assay was performed at 24 h following treatment with various concentrations (0-40 µM) of piceatannol in the presence or absence of TNF-α, regardless of the presence of PBS. Piceatannol alone, or in conjunction with TNF-α, was not cytotoxic at any of the concentrations tested in this study (Fig. 1A). Therefore, piceatannol levels of <40 µM were selected for subsequent experiments. Subsequently, the effect of piceatannol on cell viability and cytotoxicity was analyzed in detail. According to the percentages of sub-G1 DNA content as measured by flow cytometry, no apoptotic cell death was observed compared with the positive H_2O_2-treated group (Fig. 1B). Furthermore, DNA fragmentation was analyzed to determine the level of fragmented DNA. No fragmented DNA was identified in the piceatannol alone or the TNF-α-treated groups compared with the positive H_2O_2-treated group (Fig. C). To determine whether piceatannol also inhibited TNF-α-induced migration, a scratch wound healing assay was performed. Cell migration was significantly increased by TNF-α treatment and was inhibited by the presence of piceatannol (Fig. 1D). Overall, these data indicate that piceatannol inhibited TNF-α-induced migration, and hence tumor invasion, without any cytotoxicity.

**Piceatannol inhibits TNF-α-induced invasion of DU145 cells by suppressing MMP-9 activation.** Zymography, RT-PCR and measurement of luciferase reporter activity were conducted to assess whether piceatannol regulates TNF-α-induced MMP-9 expression. The zymography data revealed that the secretion of MMP-9 was significantly increased by TNF-α treatment compared with the untreated control group.
Piceatannol decreased TNF-α-induced MMP-9 activity in a dose-dependent manner (Fig. 2A). In a subsequent experiment, the luciferase activity of MMP-9 was assayed using transient transfection with reporter vectors that included MMP-9 promoters. Treatment with TNF-α significantly increased MMP-9 luciferase activity, whereas piceatannol downregulated TNF-α-induced MMP-9 luciferase reporter activity (Fig. 2B). In addition, RT-PCR analysis revealed that steady-state MMP-9 mRNA levels are undetectable in piceatannol-treated cells compared with the untreated control group (Fig. 2C). TNF-α stimulation of cells resulted in a significant increase in MMP-9 mRNA expression compared with the untreated control; however, piceatannol completely reversed TNF-α-induced MMP-9 mRNA levels to the levels of the untreated control. Since MMP-9 is thought to be critically involved in the processes of tumor invasion (25), the effects of piceatannol on the invasion of DU145 cells was examined. In the invasion assay, when the cells were treated with TNF-α alone, a marked 4-fold increase in cell invasion was observed compared with the untreated control. However, the addition of piceatannol resulted in an ~50% reduction in penetration through a Matrigel-coated membrane when compared with the TNF-α-treated group (Fig. 2D). Addition of MMP-9 inhibitor I significantly blocked TNF-α-induced cell invasion. These results confirm that piceatannol suppressed the upregulation of TNF-α-stimulated MMP-9 expression at the transcription level and inhibited TNF-α-induced invasion of DU145 carcinoma cells.

Piceatannol inhibits TNF-α-induced expression of the MMP-9 gene by suppression of NF-κB activity. In order to determine whether MMP-9 mRNA expression was linked to NF-κB activity, a promoter assay was conducted using DU145 cells that had been transiently transfected with a luciferase reporter vector, which included the NF-κB-binding sites. NF-κB luciferase activity was significantly increased, ~7.4-fold, in TNF-α-stimulated DU145 cells compared with the untreated control group (Fig. 3A). However, the TNF-α-stimulated luciferase activity in the cells containing the NF-κB construct was significantly reduced to ~50%, by treatment with piceatannol. An EMSA was conducted to evaluate the effect of piceatannol on the DNA-binding activity of NF-κB to assess its inhibitory effect on the transcriptional activity of MMP-9. Nuclear extracts from control and piceatannol-treated DU145 cells were subjected to analysis for the DNA-binding activity of NF-κB. This reached a maximum at 30 min and was sustained for 1 h following treatment with TNF-α (data not shown). EMSA data revealed that treatment with piceatannol downregulated the TNF-α-induced DNA-binding activity of NF-κB at 30 min (Fig. 3B). As shown in the western blot analysis, treatment with TNF-α decreased the levels of p65 and p50 in the cytosolic extract, whereas the addition of piceatannol resulted in sustainment of the levels of p65 and p50. This suggests that piceatannol inhibits the nuclear translocation of NF-κB subunits p65 and p50 (Fig. 3C). In addition, our data indirectly demonstrated that a specific NF-κB inhibitor PDTC has the ability to inhibit TNF-α-induced expression of MMP-9 mRNA (Fig. 3D). These results indicate that treatment with piceatannol suppresses MMP-9 gene expression in TNF-α-induced DU145 cells via inhibition of NF-κB activity.

Piceatannol downregulates Akt phosphorylation, which regulates NF-κB-dependent MMP-9 gene expression. As Akt is known to be an upstream regulator of NF-κB, we investigated whether NF-κB activity is regulated by piceatannol-induced Akt phosphorylation or dephosphorylation. Western blot analysis revealed that TNF-α stimulation induced the
phosphorylation of Akt at 15 min, whereas treatment with piceatannol suppressed the phosphorylation of Akt (Ser 473, Fig. 4A). It was also demonstrated that a specific Akt inhibitor LY294002 completely eliminated the TNF-α-induced NF-κB promoter activity, suggesting that inactivation of Akt by piceatannol regulates TNF-α-induced NF-κB (Fig. 4B).
Moreover, RT-PCR analysis demonstrated that pretreatment of DU145 cells with LY294002 suppressed TNF-α-induced MMP-9 activity at the transcription level (Fig. 4C). These results confirmed that piceatannol regulated TNF-α-induced MMP-9 gene expression by blocking Akt phosphorylation-dependent NF-κB activity.

**Discussion**

Our previous study determined that piceatannol considerably suppresses lipopolysaccharide-induced pro-inflammatory cytokines and nitric oxide expression in BV2 microglial cells (15). Furthermore, it has been reported that piceatannol enhances TRAIL-induced apoptosis in human leukemia THP-1 cells via Sp1- and ERK-dependent DR5 upregulation (16). It is not fully known how piceatannol regulates anticancer activity during the invasion process. Therefore, we investigated whether piceatannol inhibits TNF-α-induced MMP-9, leading to decreased invasion of DU145 prostate cancer cells, by suppression of NF-κB activity. This study provides substantial evidence that piceatannol inhibits TNF-α-induced MMP-9 expression in DU145 prostate cancer cells by suppressing Akt-mediated NF-κB activity.

MMPs are capable of digesting various components of the extracellular matrix and other molecules, including cell surface receptors, growth factors and cell adhesion molecules (17,18). Thus, they play a significant role in tissue repair, tumor invasion and metastasis (19,20). The generation and analysis of transgenic and knockout mice for MMPs have confirmed that MMPs play key roles in the process of carcinogenesis and invasion (21). One of these, MMP-9, is regarded as a critical molecule in tumor progression and invasion (22). Therefore, inhibition of MMP-9 appears to be an ideal strategy to control tumor growth, invasion and metastasis. Our results demonstrate that piceatannol inhibits TNF-α-induced MMP-9 activity accompanied by the suppression of MMP-9 gene transcription in DU145 prostate cancer cells. Notably, the Matrigel assay revealed that piceatannol significantly suppresses cell invasion when compared with an MMP-9 inhibitor. Previous studies have also identified the signal transduction pathways involved in regulating MMP-9 expression in various tumor cells (23,24). In particular, NF-κB is a significant transcription factor for regulating the MMP-9 gene promoter and contains NF-κB-binding sites (12). This study revealed that piceatannol regulates NF-κB activity by inhibiting p65 and p50 protein translocation. The results of the current study suggest that the downregulation of NF-κB by piceatannol potentiates antitumor and antimetastatic activities via the downregulation of MMP-9 expression. A previous study has illustrated that the suppression of NF-κB activity in human prostate cancer cells inhibits their tumorigenic and metastatic properties in nude mice by suppressing angiogenesis and invasion via the downregulation of MMP-9 (25). It has also been reported that several transcription factors in the human MMP-9 promoter region, including AP-1 and Sp1, regulate MMP-9 expression in response to PMA and TNF-α induction (26,27). Therefore, further studies are required to determine whether other transcriptional factors are inhibited in piceatannol-induced MMP-9 downregulation. In addition, we investigated whether piceatannol inhibits Akt phosphorylation, as Akt is the main molecule upstream of NF-κB that has a significant role in cellular growth, adhesion and differentiation (28). Treatment with piceatannol completely suppressed Akt phosphorylation. This was further confirmed by conducting luciferase reporter assays and RT-PCR following treatment of the cells with an Akt inhibitor LY294002. Therefore, the NF-κB-mediated Akt pathway revealed the absolute effects on TNF-α-induced MMP-9-dependent invasion in this study.

The role of MAPKs in the regulation of MMP-9 expression is particularly well understood in TNF-α-stimulated cancer cells (29-31). Previously, the regulation of TNF-α-induced MMP-9 activity has been reported via ERK, p38 and JNK phosphorylation, whereas non-phosphorylated ERK, p38 and JNK kinase expression was unaffected (32). However, Lee et al have reported that TNF-α-induced p38 is an effector MAPK that induces MMP-9 expression regardless of the activation of ERK1/2 and JNK (23). Therefore, additional studies are necessary to elucidate the precise signaling mechanism that controls NF-κB activity and its regulatory kinases during piceatannol-induced MMP-9 inhibition in DU145 prostate cells.
In summary, the current study suggests that MMP-9 plays a considerable part in the regulation of MMP-9, resulting in an inhibition of invasion in DU145 prostate cancer cells. Finally, these results demonstrate that piceatannol is a potent inhibitor of TNF-α-induced MMP-9 expression and invasion by suppressing the Akt-mediated NF-κB pathway.

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References