Bee venom suppresses PMA-mediated MMP-9 gene activation via JNK/p38 and NF-κB-dependent mechanisms

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A R T I C L E   I N F O

Article history:
Received 12 October 2009
Received in revised form 1 December 2009
Accepted 1 December 2009
Available online 5 December 2009

Keywords:
Bee venom
Phospho-p38
Phospho-JNK
NF-κB
MMP-9
MCF-7 cells

A B S T R A C T

Ethnopharmacological relevance: Bee venom has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and for the relief of pain in traditional oriental medicine.

Aim of the study: The purpose of this study is to elucidate the effects of bee venom on MMP-9 expression and determine possible mechanistic mechanisms by which bee venom relieves or prevents the expression of MMP-9 during invasion and metastasis of breast cancer cells. We examined the expression and activity of MMP-9 and possible signaling pathway affected in PMA-induced MCF-7 cells.

Material and methods: Bee venom was obtained from the National Institute of Agricultural Science and Technology of Korea. Matrigel invasion assay, wound-healing assay, zymography assay, western blot assay, electrophoretic mobility shift assay and luciferase gene assay were used for assessment.

Results: Bee venom inhibited cell invasion and migration, and also suppressed MMP-9 activity and expression, processes related to tumor invasion and metastasis, in PMA-induced MCF-7 cells. Bee venom specifically suppressed the phosphorylation of p38/JNK and at the same time, suppressed the protein expression, DNA binding and promoter activity of NF-κB. The levels of phosphorylated ERK1/2 and c-Jun did not change. We also investigated MMP-9 inhibition by melittin, apamin and PLA2, representative single component of bee venom. We confirmed that PMA-induced MMP-9 activity was significantly decreased by melittin, but not by apamin and phospholipase A2. These data demonstrated that the expression of MMP-9 was abolished by melittin, the main component of bee venom.

Conclusion: Bee venom inhibits PMA-induced MMP-9 expression and activity by inhibition of NF-κB via p38 MAPK and JNK signaling pathways in MCF-7 cells. These results indicate that bee venom can be a potential anti-metastatic and anti-invasive agent. This useful effect may lead to future clinical research on the anti-cancer properties of bee venom.

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1. Introduction

The invasion and metastasis of cancer cells are known to be the primary causes of cancer progression (Weng et al., 2008). These are complicated processes involving a group of proteolytic enzymes, which participate in the degradation of tissue barriers such as the extracellular matrix (ECM) and basement membrane (Kato et al., 2002; Liotta et al., 1991). Among the group of proteolytic enzymes, matrix metalloproteinases (MMPs) play an important role in tissue repair, angiogenesis, apoptosis, tumor invasion, and metastasis (Rahman et al., 2006; Sternlicht and Werb, 2001). MMPs, which belong to a large family of 24 highly homologous, zinc-dependent, ECM-degrading proteases, can be divided into collagenase, stromelysins, matrilysins and gelatinases (Hong et al., 2005b). One of the members of MMP family, the type IV collagenase (MMP-9) is critical for cell migration and can lead to invasion and metastasis of cancers (Nabeshima et al., 2002). The increase in activity and expression of MMP-9 has been frequently observed in many human cancers with invasive and metastatic capability (Basset et al., 1997; Johnsen et al., 1998). In clinically invasive breast cancer, augmented protein levels and activity of MMP-9 was associated with shortened patient survival and unfavorable prognosis (Rouyer et al., 1994). Also, previous studies have shown that MMP-9 induces inhibition of apoptosis in medulloblastoma cells (Bhoopathi et al., 2008). MMP-9 has been shown to be highly expressed in various types of cancer, including breast cancer.
expressed and strongly correlated with the malignant phenotype in various cancers. The expression of MMP-9 is adjusted by various physical stimulators, growth factors (fibroblast growth factor-2; FGF-2), epidermal growth factor; EGF and hepatocyte growth factor; HGF) and cytokines (tumor necrosis factor-alpha; TNF-α) (Gum et al., 1997; Hozumi et al., 2001; Weng et al., 2008; Zeigler et al., 1999), or chemical, phorbol-12-myristate-13-acetate (PMA) (Cho et al., 2007). Among these stimulators, PMA can act as a tumor promoter that induces MMP-9 expression in certain cancer cells (Cho et al., 2007). The expression of growth factor or cytokines-induced MMP-9 is modulated by the activation of various transcription factors such as activator protein-1 (AP-1) (Gum et al., 1997) and nuclear factor-kappaB (NF-κB) (Eberhardt et al., 2000; Hong et al., 2005a; Lee et al., 2008) through Ras/Raf/MEK/ERK (Cho et al., 2007), c-Jun N-terminal kinase (JNK), phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB) (Sato et al., 2002) and p38 MAPK signaling pathway (Lee et al., 2008).

Bee venom has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and for the relief of pain in oriental medicine (Kwon et al., 2001a,b). Bee venom consists of many biologically active enzymes, peptides and biogenic amines, such as melittin (the major active ingredient of bee venom), apamin, adolapin and mast cell-degranulating peptide (Park et al., 2004; Tu et al., 2008). Bee venom was reported to cause growth arrest or exhibited cytotoxic effects in hepatocarcinoma cells (Chu et al., 2007; Li et al., 2006). Also, proliferation of melanoma cells, activity of apoptotic enzyme (bcl-2 and caspase-3) in leukemic cells and ERK and Akt signaling pathway of renal cancer cells were all regulated and/or suppressed by bee venom (Moon et al., 2006; Son et al., 2007; Liu et al., 2002; Jang et al., 2003). Recent studies have reported that bee venom-induced apoptosis in breast cancer cell line and lung cancer cell line (Son et al., 2007). The promotion of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspases and MMP-9, is essential for the bee venom and melittin-induced anti-cancer effects (Holle et al., 2003; Moon et al., 2006; Rahman et al., 2006; Bhooopathi et al., 2008). However, regulation of underlying mechanisms of MMP-9, main factor to apoptosis, invasion and metastasis of cancer cells, by bee venom are largely unknown. Also, the molecular mechanisms by which bee venom acts on the expression of MMP-9 and its effect upon the invasiveness of breast cancer cells, MCF-7, which have highly invasive, are still undefined.

In this study, PMA-induced MCF-7 cells were used to study the effects of bee venom on MMP-9 expression and to determine possible mechanisms on how bee venom relieves or prevents expression of MMP-9 in breast cancer cells. We showed that expression of PMA-induced MMP-9 suppresses by bee venom.

2. Materials and methods

2.1. Cells culture and bee venom

Human MCF-7 cells were obtained from the American type culture collection (Rockville, MD). The culture medium used in the experiments was Dulbecco’s modified Eagle’s medium-low glucose (Gibco-BRL, Gran Island, NY, USA), containing 10% fetal bovine serum (Gibco) and 1% antibiotics (Gibco). Bee venom was obtained from the National Institute of Agricultural Science and Technology (NIAST), Suwon, Korea.

2.2. Cytotoxicity assay

The cells were grown to 70% confluence and treated with bee venom for 24 h, and the cell viability was determined by MTT assay. 3(4,5-dimethylthiazol2-yI)-2,5-diphenyltetra-zolium bromide (MTT) (Roche Applied Science, Indianapolis, IN, USA) was added 4 h prior to the termination of the culture. The amount of formazan deposits was quantified according to the supplier’s protocol.

2.3. Matrigel invasion assay

Cell invasion assay were carried out as previously reported (Chung et al., 2004) with some modifications, and 5 × 10^5 cells per chamber were seeded in each invasion assay. The upper insert of a transwell (Corning Costar, Cambridge, MA) was coated with 30 μl of a 1:2 mixture of matrigel/PBS. The cells were plated on the matrigel-coated upper chamber, and the media of presence or absence of drugs added to the upper chamber of the transwell insert. The lower chamber was filled culture medium. Cells in the chamber were incubated for 24 h at 37°C and cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted by light microscopy.

2.4. Wound-healing assay

Wound-healing assay was performed using the procedure described by Lin et al. (2008) with minor modification. The MCF-7 cells were seeded in 6-well plate and incubated until they reached 80% confluence. The monolayer cells were scratched with a 200 μl pipette tip to create a wound, and cells were washed twice with serum-free culture media to remove floating cells and then replaced with fresh medium without serum. Cells were subjected to the indicated treatment for 24 h, and cells migrating from the leading edge were photographed at 0 and 24 h.

2.5. Zymography assay

The resultant supernatant was subjected to SDS-PAGE containing 10% polyacrylamide and 1 mg/ml of gelatin. After electrophoresis, run at 4 °C, the gels were washed several times with 2.5% Triton X-100 for 30 min at room temperature and incubated overnight at 37 °C in buffer containing 5 mM CaCl2 and 1 M ZnCl2. The gels were stained with coomassie brilliant blue R-250 (0.1% coomassie brilliant blue R-250, 45.5% methanol, 9% acetic acid) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 30 min, and then destained for 1 h in a solution of 10% acetic acid and 10% methanol.

2.6. Western blot analysis

Cell total lysates prepared in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% nonidet P-40, 100 ml phenylmethylsulfonyl fluoride, 20 mM aprotinin, and 20 mM leupeptin, adjusted to pH 8.0) at 4 °C for 30 min, followed by centrifugation at 13,000 rpm for 5 min. Cytosol/nucleo extraction, cultured cells were collected by centrifugation, washed, and suspended in buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride]. After 15 min on ice, the cells were vortexed in the presence of 0.5% nonidet P-40. The nuclear pellet was then collected by centrifugation and extracted with buffer B [20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] for 15 min at 4 °C. After cytosolic extraction, the membrane proteins in the pellet were extracted with buffer C [200 mM Tris–HCl (pH 7.5), 1% nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] for 30 min on ice and centrifuged. The supernatant was saved as a detergent-soluble membrane fraction. The cell extract was subjected on SDS-PAGE, electrottransferred to immobilon-P.
membranes (Millipore, Cork, Ireland). Western blotting was carried out as described before (Cho et al., 2007; Hong et al., 2005b). Detection of specific proteins was carried out by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA), following to the manufacturer’s instruction. The phospho-JNK, total-JNK, phospho-p38, total-p38, phospho-ERK1/2 and total-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., CA, USA). NF-κB and c-jun antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology Inc., Beverly, USA).

2.7. RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen Co, Grand Island, NY, USA) according to manufacturer’s description. The quantity and purity of the isolated RNA were measured at OD260 and OD280. Total RNA was reverse-transcribed into cDNA by oligo(dT) priming using moloney murine leukaemia virus reverse transcriptase (all purchased from Promega, Madison, WI, USA). cDNA was amplified by PCR using primer for MMP-9; forward 5′-GGGACACAGGAGCCTGTA-3′; reverse 5′-TGAGGGGAGAAGCAACC-3′, TIMP-1; forward 5′-CTGTTGTCCTGTCCTGATA-3′; reverse 5′-CCGGTGACGGCCAAATGAC-3′, TIMP-2; forward 5′-GATGTGACGGCCCAAGAAG-3′, reverse 5′-CTCTGTACCACAGGTCA-3′; and β-actin, forward 5′-GCCATCGTCACCAACTGGGAC-3′, reverse 5′-CTGTTGTTGCTGTGGCTGATA-3′. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

2.8. Electrophoretic mobility shift assay

Cultured cells were collected by centrifugation, washed and suspended in buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF], After 15 min on ice, the cells were vortexed in the presence of 0.5% nonidet P-40. The nuclear pellet was then collected by centrifugation and extracted with buffer B [20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF] for 15 min at 4 °C. The nuclear extracts (10 μg) were incubated at 4 °C for 30 min in 25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol with 1 μg of poly dI/dC and 5 pmol of a probe end-labeled with 32P-ATP, and resolved by electrophoresis at 4 °C in 6% poly-acrylamide gels using a TBE running buffer. Probes included 25 mer oligonucleotides encompassing the consensus sequences for AP-1 and NF-κB. Gels were rinsed with water, dried and exposed to X-ray film overnight.

2.9. Plasmid transfection and luciferase gene assays

pGL2-MMP-9-WT, pGL2-MMP-9-mAP-1-2 and pGL2-MMP-9-mNF-κB were used in transient transfection assays. Cells were plated onto 6-well dishes at a density of 1 × 10^4 cells/ml and allowed to grow overnight. The cells were cotransfected with various plasmid constructs and the pCMA-β-galactosidase plasmid for 12 h with DNA transfection reagent (TransIT-LT1 transfection reagent, Mirus, Madison, WI, USA) according to the manufacturer’s instructions. After 12 h incubation in fresh medium, the enzyme activities of luciferase and β-galactosidase were determined using commercial kits (Promega), according to the manufacturer’s protocol. Luciferase activity was calculated as luciferase activity normalized with β-galactosidase activity in each cell lysate.

2.10. Statistical analysis

All in vitro results are representative of at least three independent experiments performed in triplicate; p < 0.05, statistically significant between experimental and control values. Significance of difference between experimental and control values was calculated using analysis of variance with Newman–Keuls multi-comparison test.

3. Results

3.1. Bee venom inhibits cell invasion and migration of MCF-7 cells

Because bee venom is poisonous in tissues and to both normal and cancerous cells (Lee et al., 2007; Tu et al., 2008), we first evaluated the effect of bee venom on the viability of MCF-7 cells by MTT assay. Bee venom decreased cell viability from 0 to 80% in a dose-dependent manner (Fig. 1A), and it showed significant cytotoxicity towards MCF-7 cells. Based on these results, non-cytotoxic concentrations of bee venom, ranging from 1 to 3 μg/ml, were used for further examination. Transwell and wound-healing assays were performed to investigate the effect of bee venom on invasion and
migration of MCF-7 cells. The invasion (Fig. 1B) and wound-healing assays (Fig. 1C) showed that invasion and migration of MCF-7 cells were increased by PMA when compared with PMA-untreated control cells. Bee venom at 3 μg/ml almost abrogated PMA-induced MCF-7 cells invasion and migration. These results suggest that bee venom has anti-tumor effects through the suppression of PMA-promoted cell invasion and migration.

3.2. Bee venom suppresses PMA-induced MMP-9 activity and expression

MMP-9 was shown to be highly expressed and strongly correlated with various cancers including breast cancer. The effects of bee venom on the PMA-promoted MMP-9 activity and expression were examined by zymography assay and western blot. Both the secretion of MMP-9 in the conditioned medium of MCF-7 cells and gene expression of MMP-9 were significantly induced by PMA at 50 nM (Fig. 2A upper and middle panels, and 2B upper panel), whereas with bee venom treatment, the level of MMP-9 decreased in a dose-dependent manner (Fig. 2A and B). Since MMP-9's activity and expression is tightly regulated by its endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs) (Hong et al., 2005b; Lee et al., 2008), we further examined the transcription level of TIMP-1 and TIMP-2 in the cells was analyzed by RT-PCR. The β-actin expression was included as an internal control.

3.3. Bee venom inhibits PMA-induced activation of MAP kinase pathway

Mitogen-activated protein kinases (MAPKs) are the most important signaling molecules involved in regulating the synthesis and release of MMP-9 by various PMA-induced cell lines (Cho et al., 2007). Therefore, it is possible that bee venom may inhibit PMA-promoted MMP-9 expression by down-regulation of the MAPK signaling pathway. To evaluate the effects of bee venom on these signaling cascades, we used antibodies against the phosphorylated forms of the three MAPKs including JNK, p38, and ERK1/2. Bee venom specifically suppressed phosphorylation of p38 and JNK, whereas the level of phosphorylated ERK1/2 was increased (Fig. 3A). Also, as shown in Fig. 3B, the expression of p65, NF-κB subunit, was dramatically suppressed after bee venom treatment while c-Jun expression did not change (Fig. 3B). These results indicate that the main targets of bee venom for its inhibitory effects on the activity and expression of MMP-9 are p38/JNK and NF-κB.

3.4. Bee venom specifically suppresses NF-κB activity

The MMP-9 promoter contains two transcriptional elements, which are the binding sites of AP-1 and NF-κB. To evaluate the activation of NF-κB and AP-1 in PMA-promoted MMP-9 expression, we investigated the change in DNA-binding activity of NF-κB and AP-1 caused by bee venom. As shown in Fig. 4A, DNA-binding activity of AP-1 and NF-κB significantly increased with PMA induction, and the NF-κB activity induced by PMA dramatically decreased when cells were treated with bee venom. However, DNA-binding activity of AP-1 did not change with bee venom treatment (Fig. 4A). To confirm the results of the EMSA analysis, reporter gene assay was performed in parallel, using reporter vec-
Fig. 4. Bee venom specifically suppressed NF-κB activity induced by PMA. (A) After 24 h of starvation in serum-free media, MCF-7 cells were incubated with bee venom for 1 h, and were treated with 50 nM PMA for 1 h. After the treatment for 1 h, the cells were collected, and nuclear extract prepared from control or MCF-7 cells treated with 50 nM PMA and bee venom was mixed with radioactive oligonucleotides containing AP-1 and NF-κB motif of the MMP-9 promoter. Bound complexes were analyzed by electrophoresis. (B) Mutations were introduced in the NF-κB or AP-1 binding sites of pGL2-MMP-9-WT (2 bp changes). MCF-7 cells were transfected with pGL2-MMP-9-WT, pGL2-MMP-9-mNF-κB, pGL2-MMP-9-mAP-1-2 reporter plasmids for 24 h. MCF-7 cells were cultured with bee venom and/or PMA for 24 h, and the relative luciferase activity in the cell extract was determined. The values are mean ± S.E. of three independent experiments at *p < 0.05 vs. control.

Fig. 5. Inhibition of MMP-9 activity by a single bee venom component, melittin. MCF-7 cells were incubated with individual bee venom component, melittin, apamin, PLA2, and/or PMA (50 nM) for 24 h in serum-free media. MMP-9 activity in the medium was analyzed by zymography assay.

3.5. Melittin suppresses PMA-induced MMP-9 activity and expression

Bee venom suppressed PMA-mediated MMP-9 gene activation via JNK, p38 MAPK and NF-κB-dependent mechanisms (Figs. 3 and 4). Because bee venom is composed of many chemical agents, we investigated MMP-9 inhibition activity of melittin, apamin and PLA2, representative single compound of bee venom, by zymography assay. As shown in Fig. 5, PMA-induced MMP-9 activity was significantly decreased by melittin, but not apamin and phospholipase A₂ (PLA₂). Consequently, we suggest that melittin is the main bee venom component that inhibits MMP-9 activity. These data demonstrated that the expression of MMP-9 was abolished because of melittin, which is the main component of bee venom.
4. Discussion

Bee venom has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and for the relief of pain in oriental medicine (Jang et al., 2003). It is composed of various peptide and non-peptide components with the peptides composed mainly of apamin, melittin and PLA₂ (Jang et al., 2005). The non-peptide components consist of histamine, dopamine and nora- drenalin (Lee et al., 2001). In this study, we examined whether bee venom can regulate MMP-9 activity and expression, which are related to tumor invasion and metastasis, in PMA-induced MCF-7 cells. MMP-9 specifically degrades type IV collagen, the main component of the ECM, and appears to play a crucial role in tumor invasion and metastasis (Ioth et al., 1998; Johnsen et al., 1998; Hong et al., 2005a). We found that bee venom directly inhibits the invasive and migratory ability of MCF-7 cells via the suppression of MMP-9 expression without abolishing the expression of TIMP-1 and -2 (Figs. 1 and 2). Bee venom effectively suppressed PMA-induced MMP-9 gene expression through the suppression of p38/JNK and NF-κB expression resulting in decreased invasion and migration of MCF-7 cells (Fig. 3). However, bee venom led to an increase in phosphorylation of ERK1/2 (Fig. 3). MAPKs, including ERK, JNK and p38, has been implicated in the control of several diverse biological processes, such as cell proliferation, differentiation and apoptosis, and has complementary cooperation on cell survival (Stone and Chambers, 2000; Takada et al., 2004). In this study, activation of ERK1/2 might protect for only cell survival (Tu et al., 2008), while bee venom arrested PMA-induced cell growth and invasion by suppression of MMP-9 expression via inhibition of phosphorylation of p38 MAPK and JNK.

PMA controls the expression of MMP-9 by modulating the activity of various transcription factors such as AP-1 and NF-κB through Ras/Raf/ERK, P38 MAPK, JNK and PI3K/PKB signaling pathways (Cho et al., 2007; Chung et al., 2004; Hong et al., 2005b). It has been demonstrated that PMA-induced MMP-9 is regulated through the ERK1/2 and AP-1 signaling pathway in Caki-1, human renal carcinoma cells and U2OS, human osteosarcoma cells (Cho et al., 2007; Hong et al., 2005b). In this paper, bee venom dramatically reduced the PMA-induced phospho-p38, phospho-JNK expression and DNA binding activity of NF-κB in a dose-dependent manner, whereas it did not affect the PMA-induced binding activity of AP-1 (Fig. 4). The results reconfirmed that treatment of cells with a dose-dependent manner of bee venom do not decrease PMA-promoted luciferase activity of pGLO2-MMP-9-nFB. These data clearly show that bee venom regulates the transcriptional activation of MMP-9 through the specific inhibition of PMA-induced p38/JNK and NF-κB activities.

The various components of bee venom have been previously described and is mostly composed of apamin (Son et al., 2007), melittin, PLA₂ (Jang et al., 2005), adolapin and mast cell-degranulating peptide (MCCP) with melittin being the major component (Son et al., 2007). We analyzed inhibitory effects of three individual bee venom component, melittin, apamin and PLA₂ on MMP-9. The enzyme activity of MMP-9 was suppressed by melittin but not by apamin and PLA₂. These results suggest that the specific inhibition of MMP-9 was regulated by a single component of the bee venom, which is melittin. In conclusion, bee venom inhibits PMA-induced MMP-9 expression and activity by inactivation of NF-κB via p38 MAPK and JNK signaling pathway in MCF-7 cells. These results indicate that bee venom is a potential anti-metastatic and anti-invasive agent and this useful effect may expand future clinical research on the anti-cancer properties of bee venom.

Acknowledgement

This work was supported by a grant (Code #: 20070301-034-001) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References


