Vanillin Inhibits Matrix Metalloproteinase-9 Expression through Down-Regulation of Nuclear Factor-κB Signaling Pathway in Human Hepatocellular Carcinoma Cells

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ABSTRACT

Vanillin has been reported to exhibit anti-invasive and antimetastatic activities by suppressing the enzymatic activity of matrix metalloproteinase-9 (MMP-9). However, the underlying mechanism of anti-invasive activity remains unclear so far. Herein we demonstrate that vanillin reduced 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 gelatinolytic activity and suppressed cell invasion through the down-regulation of MMP-9 gene transcription in HepG2 cells. Vanillin significantly reduced the 6.6-fold invasive capacity of HepG2 cells in noncytotoxic concentrations, and this anti-invasive effect was concentration-dependent in the Matrigel invasion assay. Moreover, vanillin significantly suppressed the TPA-induced enzymatic activity of MMP-9 and decreased the induced mRNA level of MMP-9. Analysis of the transcriptional regulation indicated that vanillin suppressed MMP-9 transcription by inhibiting nuclear factor-κB (NF-κB) activity. Western blot further confirmed that vanillin inhibited NF-κB activity through the inhibition of IκB-α phosphorylation and degradation. In conclusion, vanillin might be a potent anti-invasive agent that suppresses the MMP-9 enzymatic activity via NF-κB signaling pathway.

Tumor invasion and metastasis require increased expressions of matrix metalloproteinases (MMPs) (Stamenkovic, 2000). MMP family is involved in the degradation of extracellular matrix, and members of MMPs have been implicated in malignancy and metastasis (Westermarck and Kahari, 1999; Stamenkovic, 2000). Among the members of MMPs, elevated serum levels of MMP-9 were reported in patients with hepatocellular carcinoma (HCC) (Hayasaka et al., 1996). MMP-9 gene (also termed gelatinase B or 92-kDa type IV collagenase) was found to highly express in HCC with invasive potential (Arii et al., 1996). Therefore, MMP-9 is suggested to serve as a new serum marker of HCC in patients with chronic liver disease (Paradis et al., 2005). MMP-9 degrades basement membrane type IV collagen and expresses during cellular invasion and metastasis (Nelson et al., 2000). The activity of MMP-9 is tightly controlled, mainly at the transcription level (Stamenkovic, 2000). The promoter of MMP-9 is highly conserved and is shown to contain multiple functional elements, including nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) elements (Sato and Seiki, 1993).

NF-κB has been shown to regulate the expression of a number of genes, such as vascular endothelial growth factor and MMP-9, whose products are involved in tumorigenesis (Garg and Aggarwal, 2002). Several evidences indicate that the suppression of MMP-9 prevents invasion and metastasis (Cha et al., 1996). MMP-9 has been considered to be an important factor in facilitating invasion and metastasis in pancreatic cancer (Nagakawa et al., 2002). Moreover, Murono et al. (2000) demonstrated that the invasiveness induced by Epstein-Barr virus latent membrane protein 1 is correlated with the induction of MMP-9. Thus, agents that inhibit the activation of NF-κB or MMP-9 may exhibit the therapeutic potential for the suppression of carcinogenesis and tumor metastasis (Banerjee et al., 2002; Garg and Aggarwal, 2002).

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ABBREVIATIONS: MMP, matrix metalloproteinase; AP-1, activator protein 1; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; MMP-9, matrix metalloproteinase-9; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; RLU, relative luciferase unit; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of natural vanilla, which is one of the most widely used flavor component in food and personal products, with an estimated annual worldwide consumption of more than 2000 tons (Rao and Ravishankar, 2000). Besides its flavor qualities, vanillin exhibits the antimicrobial potential and has been used as a natural food preservative (Cerrutti et al., 1997). Moreover, vanillin displays the antioxidant activity through the protection of membranes against photosensitization-induced oxidative damage in rat liver mitochondria (Aruoma, 1999; Kamat et al., 2000). Vanillin inhibits chemical and physical mutagens-induced mutagenesis in both bacteria and mammalian cells (Keshava et al., 1998; Shaughnessy et al., 2006). It also displays chemopreventive effects in multiorgan carcinogenesis and hepatocarcinogenesis models in rats (Akagi et al., 1995).

Vanillin has been known to inhibit cell invasion and migration, suppress enzymatic activity of MMP-9, and reduce the numbers of lung-metastasized colonies in mice (Lirdpramanongkol et al., 2005). However, how the MMP-9 activity is regulated by vanillin is still unclear. Herein we demonstrate that the suppression of MMP-9 activity by vanillin is via the NF-κB signaling pathway in HepG2 cells.

Materials and Methods

Cell Culture and Transfection. HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Vanillin, purchased from Sigma (St. Louis, MO), was dissolved in dimethyl sulfoxide and stocked at −30°C. 12-O-Tetradecanoylphorbol-13-acetate (TPA), purchased from Sigma, was dissolved in ethanol at 0.5 mg/ml. HepG2 cells were transiently transfected with 5 μg of plasmid DNAs by SuperFect transfection reagent (QIAGEN, Valencia, CA) and then kept in a humidified incubator at 37°C with 5% CO₂.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and dissolved in phosphate-buffered saline (137 mM NaCl, 1.4 mM KH₂PO₄, 4.3 mM Na₂HPO₄, and 2.7 mM KCl, pH 7.2). Cell viability was monitored by MTT colorimetric assay as described previously (Hsiang et al., 2005). In brief, various amounts of vanillin were added to subconfluent cell monolayers for 24 h, and one-tenth volume of 5 mg/ml MTT was added to the culture medium. After a 4-h incubation at 37°C, equal volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan, and the absorbance value was measured at 570 nm using a microplate reader. Cell viability (measured as a percentage) was calculated by (optical density of vanillin-treated cells/optical density of solvent-treated cells).

Cell Invasion Assay. Cell invasion was measured using the Matrigel-coated film insert (8 μm pore size) fitting into 24-well invasion chambers (BD Biosciences, San Jose, CA) as described previously (Hsiang et al., 2007). In brief, HepG2 cells (5 × 10⁴), which were resuspended in 200 μl of DMEM, were added to the top compartment of the invasion chamber and treated with various amounts of vanillin in DMEM. Thirty minutes later, 100 ng/ml TPA was added to the medium, and the Matrigel invasion chambers were incubated at 37°C in 5% CO₂. After a 24-h incubation, the filter inserts were removed from the wells, and the cells on the top side of the filter were removed using cotton swabs. The cells in the bottom surface of the filter were stained with crystal violet, and the cell number was counted with a microscope. Invasive -fold was calculated as the total number of invasive cells in treated cells relative to untreated cells. Line represents the cell viability (expressed as a percentage), which was determined by MTT assay. Values are the mean ± S.D. of three independent experiments. **, p < 0.01; ***, p < 0.001 compared with TPA-treated cells.

Fig. 1. Effect of vanillin on the TPA-induced HepG2 cell invasion. A, cell invasion assay. HepG2 cells (5 × 10⁴) were resuspended in 200 μl of DMEM and added to the top compartments of Matrigel invasion chambers. The cells were treated with various amounts of vanillin in DMEM for 30 min and then treated with 100 ng/ml TPA. After a 24-h incubation, the total number of cells on the bottom surface of the insert chamber was stained and counted under microscope with 200x magnification. B, quantitation of cell invasion assay. Results are expressed as invasion -fold, which is presented as the total number of invasive cells in treated cells relative to untreated cells. Line represents the cell viability (expressed as a percentage), which was determined by MTT assay. Values are the mean ± S.D. of three independent experiments. **, p < 0.01; ***, p < 0.001 compared with TPA-treated cells.
by dividing the invasive cell number of vanillin-treated cells by the invasive cell number of solvent-treated cells.

**Gelatin Zymographic Analysis.** HepG2 cells were treated with various amounts of vanillin in DMEM for 30 min. TPA (100 ng/ml) was then added to cells and incubated at 37°C for 24 h. Conditioned medium was mixed with equal volume of 2X nonreducing sample buffer (0.5 M Tris-HCl, 20% glycerol, 10% SDS, and 0.2% bromphenol blue, pH 6.8) and fractionated in 7.5% polyacrylamide gels containing 1 mg/ml gelatin by electrophoresis at 90 V for 120 min at 4°C. The gels were then soaked in 2.5% Triton X-100 for 10 min at room temperature to remove SDS, incubated in incubation buffer (50 mM Tris-HCl, 10 mM CaCl$_2$, 2H$_2$O, 50 mM NaCl, and 0.05% Brij35, pH 7.6) at 37°C for 48 h, stained with 0.25% Coomassie Brilliant Blue R-250 in 40% methanol for 30 min, and finally destained with 40% methanol and 10% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin. The intensities of bands on the gels were calculated by Gel-Pro Analyzer (MediaCybernetics Inc., Bethesda, MD).

**Total RNA Isolation.** Total RNA was extracted using a RNeasy Mini kit (Qiagen) from cells treated with or without drugs. Total RNA was quantified using the Beckman DU800 spectrophotometer (Beckman Coulter, Fullerton, CA). Samples with $A_{260}/A_{280}$ ratios greater than 1.8 were accepted for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.

**Semiquantitative RT-PCR.** One microgram of total RNA was reverse-transcribed using oligo(dT)$_{15}$ primer and SuperScriptIII (Invitrogen) in a total volume of 20 μl. Two microliters of reverse-transcription mixture was subject to PCR to measure the mRNAs of MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed with Tq polymerase (Promega, Madison, WI) for 36 cycles at 92°C for 45 s, 55°C for 45 s, and 72°C for 2 min. PCR primers for MMP-9 were as follows: sense, 5'-CGATGAGGAGTTTGTTGGCTCCCTGGC-3'; antisense, 5'-AATAGTCTAAGCCCAAAGCGCGTTGGC-3'. PCR primers for GAPDH were as follows: sense, 5'-ACCCATGGGAATTCGATCCAC-3'; antisense, 5'-CCTCGCAGGGCTGCTTCACCAC-3'. The intensities of bands on the gels were calculated by Gel-Pro Analyzer (MediaCybernetics Inc.).

**Construction of MMP-9 Promoter/Reporter Plasmids.** The MMP-9 promoters, including wild-type and mutants, were provided by Douglas D. Boyd (MD Anderson Cancer Center, University of Texas, Houston, TX) and originally constructed by Hiroshi Sato (Cancer Research Medicine, Kanazawa University, Kanazawa, Japan) (Sato and Seiki, 1993). The −670 to +54 fragment of the MMP-9 promoter was inserted into the pGL3-basic vector to generate the MMP-9 promoter/reporter plasmid. Wild-type and mutant sequences of NF-κB and AP-1 were constructed by TDIG (Promega). The arrowhead indicates the 92-kDa MMP-9. Zymographic results were calculated by Gel-Pro Analyzer (MediaCybernetics Inc.).

**Luciferase Assay.** HepG2 cells were treated with various amounts of vanillin in DMEM for 30 min. TPA (100 ng/ml) was then added to cells and incubated at 37°C for 24 h. Luciferase assay was performed as described previously (Hsiang et al., 2005). Relative luciferase activity was calculated by dividing the relative luciferase unit (RLU) of MMP-9 promoter/reporter plasmid-transfected cells by the RLU of pGL3-basic-transfected cells.

**Western Blot Analysis.** HepG2 cells were treated with various amounts of vanillin in DMEM. Thirty minutes later, 100 ng/ml TPA was added to cells and incubated at 37°C for 24 h. Cells were then lysed with 250 μl of sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue, pH 6.8). The proteins (10 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, and the protein bands were then transferred electrothermally to nitrocellulose membranes. The membranes were probed with polyclonal antibodies against p65, IκB-α, phosphorylated IκB-α, and β-actin (Cell Signaling Technology, Danvers, MA). The bound antibody was detected with peroxidase-conjugated antirabbit antibody followed by chemiluminescence (ECL System; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and exposed by autoradiography.

**Statistical Analysis.** Data were presented as mean ± S.D. Student’s t test was used for comparisons between two experiments. A p value of <0.05 was considered statistically significant.

**Results**

**Vanillin Suppressed TPA-Induced Cell Invasion.** To examine whether the cell invasion was suppressed by vanillin, we seeded HepG2 cells in the invasion chamber and calculated the number of invasive cells in the presence of vanillin. As shown in Fig. 1, treatment with vanillin had no effect on cell viability by MTT assay. The invasiveness of HepG2 cells was markedly increased after TPA treatment relative to solvent-treated control. However, vanillin inhibited TPA-induced cellular invasiveness in a dose-dependent manner, approximately 50 and 85% reduction at 0.5 and 5 mM, respectively. In addition, vanillin also inhibited TPA-induced invasiveness of other hepatocytes, such as Chang liver cells and Hep3B cells (Supplementary Fig. 1). These data indicate that vanillin is capable of suppressing TPA-induced cellular invasion, and this anti-invasive effect is cell type-independent.

**Fig. 2.** Effect of vanillin on the enzymatic activity and mRNA level of MMP-9 in HepG2 cells. A, gelatin zymographic analysis. HepG2 cells were treated with various amounts of vanillin in DMEM for 30 min and then treated with 100 ng/ml TPA. After a 24-h incubation, the conditioned medium was collected and analyzed by gelatin zymography analysis (top). The arrowhead indicates the 92-kDa MMP-9. Zymographic results were quantitated and expressed as activation fold, which is presented in comparison with the intensity of MMP-9 relative to untreated cells (bottom). Values are the mean ± S.D. of three independent experiments. *, p < 0.05 compared with TPA-treated cells. B, semiquantitative RT-PCR. HepG2 cells were treated with various amounts of vanillin in DMEM for 30 min and then treated with 100 ng/ml TPA. After a 24-h incubation, total RNA was extracted, and 1 μg of total RNA was reverse-transcribed. The resulting cDNAs were then amplified by PCR. The PCR products were resolved in agarose gels and visualized with ethidium bromide. The percentage between MMP-9 and GAPDH expressions is presented at the bottom. Values are the mean ± S.D. of triplicate assays.
Vanillin Decreased Enzymatic Activity and mRNA Level of MMP-9 in HepG2 Cells. Tumor invasion requires increased expression of MMP-9. To study whether the gelatinolytic activity of MMP was inhibited by vanillin, we performed the zymographic analysis. Figure 2A shows that TPA, as expected, induced MMP-9 enzymatic activity in HepG2 cells. Vanillin suppressed TPA-induced MMP-9 enzymatic activity, with approximately 35% reduction at 5 mM. These findings suggest that vanillin reduces MMP-9 activity and, in turn, inhibits the potential of invasion.

To further determine whether the suppression of MMP-9 by vanillin resulted from the decreased mRNA level, we performed semiquantitative RT-PCR analysis. As shown in Fig. 2B, vanillin decreased the TPA-induced mRNA level of MMP-9 in a dose-dependent manner. These results indicate that vanillin suppresses TPA-induced MMP-9 activation in HepG2 cells.
Vanillin Inhibited the Transcription of MMP-9 Promoter via NF-κB-Binding Site. To investigate whether the transcriptional activity of MMP-9 was regulated by vanillin, we examined the promoter activity of MMP-9 gene by reporter assay. A genomic fragment containing the promoter region (−670 to +54) of MMP-9 gene was amplified by PCR and subcloned into pGL3-basic vector (Fig. 3A). The resulting construct was transfected into HepG2 cells. The cells were then treated with vanillin and TPA for 24 h, and the luciferase activity was measured by luciferase assay. Figure 3B shows that TPA activated the MMP-9 promoter at a level approximately 2.5-fold greater than the pGL3-basic-transfected cells. TPA-activated MMP-9 transcription was significantly suppressed by vanillin in a dose-dependent manner. These findings suggest that vanillin inhibits the transcription of MMP-9 promoter.

Several studies indicate that the expression of MMP-9 is regulated by transcription factors, such as AP-1 and NF-κB (Sato and Seiki, 1993). Thus, the reporter plasmids with single-site mutations in the NF-κB- or AP-1-binding site of the MMP-9 promoter were constructed (Fig. 4A), and the promoter activity was analyzed by luciferase assay. As shown in Fig. 4B, vanillin significantly inhibited the NF-κB-containing promoter activity but slightly inhibited the AP-1-containing promoter activity. No cytotoxicity was observed when cells were exposed to vanillin (data not shown). These data suggest that vanillin suppresses MMP-9 gene transcription mainly via the NF-κB binding site.

To further determine the simplicity of promoter structure used by vanillin, we transfected HepG2 cells with pNF-κB-Luc or pAP-1-Luc plasmid DNAs, which contain the luciferase gene driven by NF-κB (5′-GGGGACTTTCC-3′)ₙ (n = 5) or AP-1 (5′-TGACTAA-3′)ₙ (n = 7) responsive elements, respectively. Transfected HepG2 cells were treated with TPA and vanillin for 24 h, and the luciferase activity was determined by luciferase assay. As shown in Fig. 5, TPA activated NF-κB- and AP-1-containing promoters, at levels of approximately 6- and 4.5-fold, respectively. Vanillin suppressed TPA-activated NF-κB-containing promoter in a dose-dependent manner, whereas vanillin slightly inhibited the TPA-induced AP-1-containing promoter. No cytotoxic effect was observed when cells were treated with vanillin. These findings suggest that vanillin suppresses the transcription of MMP-9 gene via NF-κB signaling pathway and, in turn, suppresses the invasiveness of HepG2 cells.

Vanillin Inhibited IκB-α Phosphorylation and NF-κB Translocation in HepG2 Cells. The activation of NF-κB is preceded by translocation of NF-κB to the nucleus after the
phosphorylation and degradation of IκB-α (Barnes and Karin, 1997). To further investigate how the NF-κB signaling pathway was affected by vanillin, we determined the levels of p65 and IκB-α proteins in vanillin-treated HepG2 cells by Western blot analysis. As shown in Fig. 6, TPA increased the nuclear translocation of p65, whereas vanillin suppressed TPA-induced p65 translocation. Because IκB phosphorylation and degradation is a predominant pathway for NF-κB activation (Karin and Greten, 2005), we next determined the levels of IκB-α proteins in cellular extracts. Phosphorylation and degradation of IκB-α was stimulated by TPA, whereas vanillin suppressed TPA-induced phosphorylation and degradation of IκB-α. The increased IκB-α level was correlated with a constant decrease of phosphorylated IκB-α in HepG2 cells. In addition, the consistent level of β-actin suggested that the reduced levels of IκB-α resulted from vanillin treatment. Therefore, these findings demonstrate that vanillin suppresses TPA-induced NF-κB activation through the inhibition of IκB-α phosphorylation and degradation.

Discussion

Approximately 70% of drugs used for the treatment of cancer are derived or based on natural products (Newman et al., 2002). Many phytochemicals are able to inhibit tumor metastasis or cell invasion through the suppression of enzymatic activities or gene expressions of MMPs (Aggarwal and Shishodia, 2006). Several dietary agents, such as curcumin, resveratrol, ursolic acid, gingerol, evodiamine, green tea catechins, and lycopene, are natural chemopreventive agents that have been found to be potent inhibitors of NF-κB (Aggarwal and Shishodia, 2006). For example, curcumin interferes with the activities of MMP-2 and MMP-9, reducing the degradation of extracellular matrix, which forms the basis of angiogenic switch (Chen et al., 2004). Moreover, lycopene decreases the secretion of MMP-9 via the NF-κB signaling pathway, leading to the inhibition of SK-Hep-1 cell invasion (Huang et al., 2007). Vanillin, at nontoxic concentrations, has been shown to inhibit the invasion and migration of cancer cells in vitro and significantly reduces the numbers of lung-metastasized colonies in mice (Lirdprapamongkol et al., 2005). However, how vanillin inhibits cellular invasion is still unclear. Therefore, we analyzed the anti-invasion mechanism of vanillin in TPA-induced HepG2 cells in this study.

TPA is one of the most well accepted agents for studying the mechanisms of carcinogenesis (Furstenberger et al., 1981). It exhibits an organotropic effect, which induces tumor promotion in skin, forestomach, mammary tissue, and lymphocytes in mice (Blumberg et al., 1982). It also exhibits many biological effects by inducing altered gene expression, a process that involves the activation of protein kinase C (Barry and Kazanietz, 2001). In addition to the carcinogenesis, TPA is able to induce MMP-9 expression via protein kinase C-dependent activation of Ras/extracellular signal-regulated protein kinase signaling pathway, resulting in the invasiveness of cell lines (Liu et al., 2002). In this study, we also demonstrated that TPA activated MMP-9 expression via the activations of AP-1 and NF-κB and induced the invasiveness of HepG2 cells. Therefore, the anti-invasive effect of vanillin was analyzed in cells exposed to TPA.

NF-κB plays central roles in promoting cancer cell motility and invasion (Baldwin 2001). Cancer progression in multiple cancers, including melanoma, breast, prostate, colorectal, and ovarian, and in specific forms of leukemia and lymphoma correlates with a significant elevation of NF-κB expression (Shattuck-Brandt and Richmond, 1997; McNulty et al., 2004). Moreover, NF-κB is involved in the regulation of inflammatory cytokine production and inflammation (Barnes and Karin, 1997). We demonstrated that vanillin suppressed TPA-induced MMP-9 expression through the inhibition of induced NF-κB activity in this study. Murakami et al. (2007) also demonstrated that vanillin inhibits lipopolysaccharide-stimulated NF-κB activation in murine macrophage cell line. These data suggest that vanillin is a potent NF-κB inhibitor that suppresses the expression of NF-κB-regulated genes.

Fig. 6. Signal transduction pathways contributing to the inhibition of NF-κB activity by vanillin in HepG2 cells. HepG2 cells treated with various amounts of vanillin in DMEM. Thirty minutes later, 100 ng/ml TPA was added to the media. The phosphorylated (phospho-IκB-α) non-phosphorylated proteins (IκB-α), and β-actin in cellular extracts were detected by Western blot. The p65 proteins in cytoplasm (cy) and nucleus (nu) were also determined by Western blot. Similar results were obtained in three different experiments.

Fig. 7. Schematic diagram illustrates the inhibitory mechanism of vanillin on the MMP-9 expression. Vanillin inhibits TPA-induced IκB-α phosphorylation and degradation, suppresses the translocation of p65, and, in turn, decreases the MMP-9 expression and the cell invasion in HepG2 cells.
Vanillin Inhibits MMP-9 Expression via NF-κB Pathway


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