

# Decursin Inhibits Induction of Inflammatory Mediators by Blocking Nuclear Factor- $\kappa$ B Activation in Macrophages<sup>S</sup>

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## ABSTRACT

In the course of screening inhibitors of matrix metalloproteinase (MMP)-9 induction in macrophages, we isolated decursin, a coumarin compound, from the roots of *Angelicae gigas*. As a marker for the screening and isolation, we tested expression of MMP-9 in RAW264.7 cells and THP-1 cells after treatment with bacterial lipopolysaccharide (LPS), the TLR-4 ligand. Decursin suppressed MMP-9 expression in cells stimulated by LPS in a dose-dependent manner at concentrations below 60  $\mu$ M with no sign of cytotoxicity. The suppressive effect of decursin was observed not only in cells stimulated with ligands for TLR4, TLR2, TLR3, and TLR9 but also in cells stimulated with interleukin (IL)-1 $\beta$ , and tumor necrosis factor (TNF)- $\alpha$ , indicating that the molecular target of decursin is common signaling mole-

cules induced by these stimulants. In addition to the suppression of MMP-9 expression, decursin blocked nitric oxide production and cytokine (IL-8, MCP-1, IL-1 $\beta$ , and TNF- $\alpha$ ) secretion induced by LPS. To find out the molecular mechanism responsible for the suppressive effect of decursin, we analyzed signaling molecules involved in the TLR-mediated activation of MMP-9 and cytokines. Decursin blocked phosphorylation of I $\kappa$ B and nuclear translocation of NF- $\kappa$ B in THP-1 cells activated with LPS. Furthermore, expression of a luciferase reporter gene under the promoter containing NF- $\kappa$ B binding sites was blocked by decursin. These data indicate that decursin is a novel inhibitor of NF- $\kappa$ B activation in signaling induced by TLR ligands and cytokines.

Macrophages are involved in the pathogenesis of inflammatory diseases such as atherosclerosis and rheumatoid arthritis. Atherosclerosis involves multiple processes, including endothelial dysfunction, inflammation, vascular proliferation, and extracellular matrix (ECM) degradation. Macrophages play particularly important roles in inflammation via the production of matrix-degrading enzymes, pro-inflammatory cytokines/chemokines, cell adhesion molecules, nitric oxide (NO), cyclooxygenase-2 (Ross, 1999). Dissolution of ECM in the fibrous cap through overexpression of active matrix metalloproteinases

(MMPs) renders this structure weak, friable, and susceptible to rupture when exposed to hemodynamic stress (Libby, 2002). Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation that leads to the destruction of cartilage and bone. Synovial inflammation involves thickening of the lining layer and infiltration of inflammatory cells into the sublining area (Cunnane et al., 1998; Vervordeldonk and Tak, 2002). In normal joints, macrophages are resident cells and cover the synovial layer. The number of macrophages in the joint greatly increases in RA synovium (Kinne et al., 2000), and the degree of increase is strongly correlated with the development of severe cartilage destruction (Yanni et al., 1994; Mulherin et al., 1996; Bresnihan, 1999). Furthermore, selective depletion of macrophages from the synovial lining before the induction of experimental arthritis resulted in prevention of both joint inflammation and cartilage destruction (van Lent et al., 1993, 1996, 1998). Joint destruction is mediated by enzymes degrading ECM, such as serine proteases, MMPs, and the

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**ABBREVIATIONS:** ECM, extracellular matrix; RA, rheumatoid arthritis; TIMP, tissue inhibitor of matrix metalloproteinase; LPS, lipopolysaccharide; PAM, palmitoyl-Cys((R,S)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH; Erk, extracellular signal-regulated kinase; INOS, inducible nitric-oxide synthase; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, threshold cycle; NF- $\kappa$ B, nuclear factor  $\kappa$ B; IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor.

cathepsins (Cunnane et al., 1998). MMP-9 levels are substantially elevated in the sera and synovial fluid from RA patients (Gruber et al., 1996; Giannelli et al., 2004). Immunohistochemistry studies have demonstrated that MMPs are expressed by cells present within atheromas but not in normal arteries (Galis, 1994; Nikkari, 1995).

Matrix metalloproteinases, a family of  $Zn^{2+}$ -dependent endopeptidases, are responsible for the degradation of most extracellular matrix proteins as well as a number of other proteins during organogenesis, growth, and normal tissue turnover. MMPs also mediate tissue remodeling in various pathologic conditions, including several inflammatory diseases (Chakraborti et al., 2003). MMP expression level is increased in atherosclerotic lesions and is linked to weakening of the vascular wall by degrading the extracellular matrix (Renko et al., 2004; Watanabe and Ikeda, 2004; Chen et al., 2005; Zeng et al., 2005). The quantity of MMP-9 (Gelatinase B, 92-kDa type IV collagenase) is low in healthy lungs but much higher in several lung diseases in which airway remodeling takes place, including asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (Holgate et al., 1999; Atkinson and Senior, 2003; Kelly and Jarjour, 2003; Suzuki et al., 2004; Whelan, 2004). In inflammatory heart diseases, MMPs have been found to play a significant role in the development of myocardial remodeling, leading to dilated cardiomyopathy and ventricular dysfunction (Pauschinger et al., 2004). In the context of neuroinflammatory diseases, including multiple sclerosis and bacterial meningitis, MMPs have been implicated in processes such as blood-brain barrier and blood-nerve barrier opening, invasion of neural tissue by blood-derived immune cells, shedding of cytokines and cytokine receptors, and direct cellular damage in diseases of the peripheral and central nervous system (Leppert et al., 2001). Infiltrating inflammatory cells are major producers of MMPs, and the paracrine/autocrine effect of a repertoire of cytokines on inflammatory cells is likely to cause an imbalance in MMP/TIMP ratio resulting, eventually, in altered extracellular matrix architecture.

To find agents that could modulate pro-inflammatory activities of macrophages, we screened extracts from 14 medicinal herbs. A coumarin compound, decursin, has been identified to be responsible for the inhibition of LPS-induced MMP-9 expression in the extract of *Angelicae gigas* radix. We further investigated the suppressive effect of decursin in the expression of MMP-9 and cytokines in cells treated with other related stimulants.

## Materials and Methods

**Plant Extracts and Purification.** For the initial screening of 14 medicinal herb extracts, EtOH extracts in the form of dried pellets were purchased from Plant Diversity Research Center (PDRC) (Daejeon, Korea). These pellets were resuspended in EtOH at 20 mg/ml concentrations and used for the initial screening. For the purification of the active compound (Supplemental Fig. 1), the dried roots of *A. gigas*, harvested in regional area, were purchased from Market of Medicinal Herbs, (Daegu, Korea), and the identity was confirmed by Dr. J. H. Yang (Daegu Haany University). The dried roots of *A. gigas* (1 kg) were extracted twice with MeOH under reflux using the methods described by Lee et al. (2002). The extracts were then combined and concentrated under low pressure to afford 21.4 g of the residue. The MeOH extract was suspended in water and then fractionated with equal volumes of  $CH_2Cl_2$  (10.5 g dry weight). The

$CH_2Cl_2$  fraction was further chromatographed on two successive silica gel columns (8 × 80 cm; Merck, Germany) and eluted with a gradient of *n*-hexane-EtOAc (20:1~8:1) to afford the active compound (1.3 g; 10:1). The structure of the compound was analyzed using  $^1H$  NMR and  $^{13}C$  NMR and confirmed to be decursin by comparison with literature values (Hata, 1966; Ryu, 1990) (Supplemental Fig. 2). The high-performance liquid chromatography was performed using Jasco system consisting of a binary pump, an autosampler, and a photodiode array detector. The ZOBAX eclipse XDB C-18 (250 × 4.6 mm) was used as column, and high-performance liquid chromatography was operated at room temperature. The eluent consisted of water with 1% acetic acid and acetonitrile with 1% acetic acid. Gradient profile was: 0~40 min from 50 to 100%. The flow rate was 1 ml/min. The wavelength of detection was 280 nm.

**Reagents and Antibodies.** Bacterial lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO); palmitoyl-Cys((R,S)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH (PAM) from Bachem AG (Bubendorf, Switzerland); poly(I)-poly(C) double strand RNA from GE Healthcare (Little Chalfont, Buckinghamshire, UK), CpG 1826 (TLR9 ligand) and CpG 2138 (control oligonucleotide) from InvivoGen (San Diego, CA), recombinant IL-1 $\beta$  and TNF- $\alpha$  from R&D Systems (Minneapolis, MN); anti-I $\kappa$ B polyclonal antibody, anti-phospho-I $\kappa$ B $\alpha$  monoclonal antibody (clone 5A5), and polyclonal antibodies to Erk1/2 and their phosphorylated forms (Thr202/Tyr204) from Cell Signaling Technology (Beverly, MA); rabbit polyclonal antibodies specific for iNOS from BD Transduction Laboratories (Lexington, KY); and monoclonal anti- $\alpha$ -tubulin clone B-5-1-2 mouse ascites fluid from Sigma.

**Cell Culture and Activation.** A murine macrophage cell line, RAW264.7 and a human macrophage cell line, THP-1, were cultured according to the supplier's instructions (American Type Culture Collection, Manassas, VA). To test the suppressive effects of herbal extracts and purified compounds on the RAW264.7 and THP-1 cells, cells were washed three times with medium (DMEM for RAW264.7 and RPMI 1640 for THP-1 cells) with 0.1% fetal calf serum and then seeded on sterile 96-well tissue culture plate ( $5 \times 10^4$  and  $1 \times 10^5$  cells/well for RAW264.7 and THP-1 cells, respectively). We then pretreated the cells with 10~30  $\mu$ g/ml herbal extracts for overnight or 20~60  $\mu$ M decursin for 2~3 h. After the pretreatment, cells were stimulated with 0.1~1  $\mu$ g/ml LPS (Sigma, St. Louis, MO) in the continued presence of decursin. Cell lysates were obtained at various time points after the activation and used for Western blotting. For the detection of MMP-9 or cytokine expression, the supernatants were collected 24 h after the activation and used for gelatin zymogram or ELISA, respectively. The BV-2 mouse microglial cell line was generously provided by Dr. E. Choi (Korea University, Seoul, Korea). The BV-2 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, 10,000 units/ml penicillin, and 10,000  $\mu$ g/ml streptomycin (Invitrogen, Carlsbad, CA) and were treated with decursin and/or stimulants in a manner similar to RAW264.7 and THP-1 cells. To obtain the peritoneal macrophages, BALB/c mice were injected i.p. (3 ml/mouse) with 3% Brewer thioglycollate medium containing 0.3 mM thioglycollate (Difco, Detroit, MI). Four days later, cell were harvested by lavage with PBS and plated into appropriate wells. Cells were allowed to adhere for 2 h and then washed free of nonadherent cells.

**Nitrite Quantification.** After BV-2 microglia cells were treated with activating agents in 96-well plates,  $NO_2^-$  concentration in culture supernatants was measured to assess  $NO$  production in microglial cells. Fifty microliters of sample aliquots were mixed with 50  $\mu$ l of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2% phosphoric acid) in a 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader.  $NaNO_2$  was used as the standard to calculate  $NO_2^-$  concentrations.

**Western Blotting, ELISA, and Gelatin Zymogram.** Cell lysates were prepared using triple-detergent lysis buffer and used for Western blot analysis as described previously (Lee et al., 2001).

Cytokine levels in the culture supernatant were measured using sandwich ELISA (R&D Systems). The detection limits were <10 pg/ml for all the cytokines. The MMP-9 activity in the culture supernatant was determined by performing substrate gel electrophoresis as described before (Lee et al., 2001).

**Cell Viability Test (MTT Assay).** To determine RAW264.7 cell viability, cells ( $5 \times 10^4$  cells/well) were washed three times with 0.1% serum DMEM and then seeded on sterile 96-well tissue culture plates. Decursin was then added at a concentration of 20–60  $\mu$ M for 3 h. After the pretreatment, the cells were stimulated with 100 ng/ml LPS (Sigma) for 24 h in the continued presence of decursin. Cell viability was evaluated in each well by the addition of 50  $\mu$ l of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 2.5 mg/ml in PBS). After 4 h of incubation, the cell-free supernatants were removed completely from each well, and 150  $\mu$ l of dimethyl sulfoxide was added. The optical densities of the wells were measured using a spectrophotometric multiwell microplate reader (Multiskan MS; Thermo Electron Corporation, Waltham, MA) at wavelength of 540 nm.

**Immunofluorescence Assay.** For the detection of intracellular location of NF- $\kappa$ B p65 subunits, RAW264.7 cells ( $2 \times 10^5$ /well in 24-well plates) were cultured in sterile cover slips and treated with Decursin and LPS as described above. At various times after the LPS treatment, the cells were fixed with 4% formaldehyde in PBS for 30 min and washed with PBS for 5 min. The fixed cells were then stained with 0.5  $\mu$ g/ml Hoechst staining solution (Sigma) for 20 min at 37°C and then washed. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at RT, washed with 0.02% Tween 20 in PBS for 20 min and 0.02% Tween 20/1% BSA in PBS for 5 min. The permeabilized cells were then treated with 2  $\mu$ g/ml anti-p65 monoclonal antibody (Santa Cruz Biotechnology) for 45 min at 37°C, washed with 0.02% Tween 20/1% BSA in PBS for 5 min. Cells were then incubated in a 1:50 dilution of Alexa Fluor 488-labeled goat anti-mouse antibody (Invitrogen) for 45 min at 37°C, and washed with 0.02% Tween 20 in PBS for 5 min and PBS for 5 min. Finally, the cover slips with cells were dried in 37°C oven for 45 min and mounted in a 1:1 mixture of xylene and malinol.

**Real-Time RT-PCR.** Five microgram of total RNAs isolated from cells were treated with RNase free DNase (BD Pharmingen, San Diego, CA), and then used to generate first-strand cDNAs using RevertAid first strand cDNA synthesis kit with 500 ng of oligo(dT)<sub>12–18</sub> primers. PCR primers were designed with ABI PRISM Primer Express 2.0 (Applied Biosystems, Foster City, CA) and made by Geno Tech Corp (Daejeon, Korea). Primers were designed for 123 and 143 base pairs for human and mouse MMP-9 and 52 and 452 base pairs for human and mouse GAPDH PCR products, respectively. Primer sequences were: human MMP-9: forward, 5'-ttctacggcactactgtgct-3'; reverse, 5'-aatcgccagtaacttccatct-3'; mouse MMP-9: forward, 5'-aaaccagacccagactctct-3'; reverse, 5'-gaggac-

acagtctgacctgaacca-3'; human GAPDH: forward, 5'-tgggctacactgagcaccag-3'; reverse, 5'-gggtgtcgtgttgaagta-3'; mouse GAPDH: forward, 5'-accacagtcctcatccac-3'; reverse, 5'-tccaccacctgtgtgtga-3'. Real-time PCR reaction was performed in an ABI PRISM 7300 sequence detector (Applied Biosystems) using SYBR green PCR mix (Applied Biosystems) with cDNA corresponding to 125 ng of original total RNA and 400 nM primers in a 20- $\mu$ l volume. The threshold cycle (Ct) values for MMP-9 reactions were normalized with Ct values from corresponding GAPDH reactions. The specificity of the PCR reaction was confirmed by control reactions, such as PCR reaction with templates processed without reverse transcriptase and PCR reaction without template. After the PCR reaction, the PCR products were run on 2% agarose gel to confirm the size and purity of the PCR products.

**Luciferase Reporter Assay.** RAW264.7 cells were plated and cultured overnight in 24-well plates ( $2 \times 10^5$  cells/well). These cells were transiently transfected with 1  $\mu$ g of plasmid DNA containing 0.1  $\mu$ g of NF- $\kappa$ B-firefly-luciferase reporter construct (containing four tandem repeats of NF- $\kappa$ B binding sites), 0.1  $\mu$ g of pRL-TK construct (containing *Renilla reniformis* luciferase gene under the thymidine kinase promoter; Promega, Madison, WI), and inert filler plasmid using SuperFect transfect reagent (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The luciferase activities were determined using the Dual-Luciferase reporter Assay System (Promega) according to the manufacturer's recommended protocol. Relative firefly luciferase activity was determined by normalization with *R. reniformis* luciferase activity.

## Results

To find agents that could suppress pro-inflammatory activities of macrophages, we screened extracts from 14 medicinal herbs (Table 1). As a marker for the screening, we tested suppression of MMP-9 induction in RAW264.7 cells stimulated with LPS. MMP-9 activity in the culture supernatant was measured using gelatin zymogram. As shown in Table 1 and Fig. 1, MMP-9 induction was suppressed by extracts of *A. gigas* radix and *Cinnamomum cassia* ramulus. In the case of extract from *C. cassia* ramulus, cytotoxicity was observed in the concentrations range used in this experiment.

Purification of the causative compounds from the roots of *A. gigas* (Supplemental Fig. 1) led to the identification of decursin (Fig. 2A). To confirm the suppressive effect of the purified compound, RAW264.7 cells were activated with LPS in the presence of decursin, which had been added 2 h before the activation. Decursin suppressed MMP-9 induction in a dose-dependent manner (Fig. 2B). Cytotoxicity was not de-

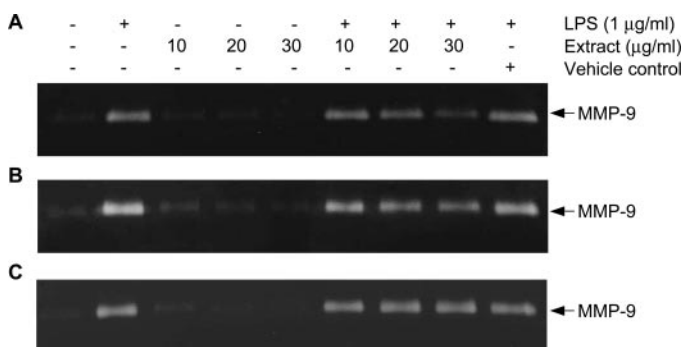
TABLE 1

List of herbal extracts used for the initial screening and their effects on LPS-induced MMP-9 expression

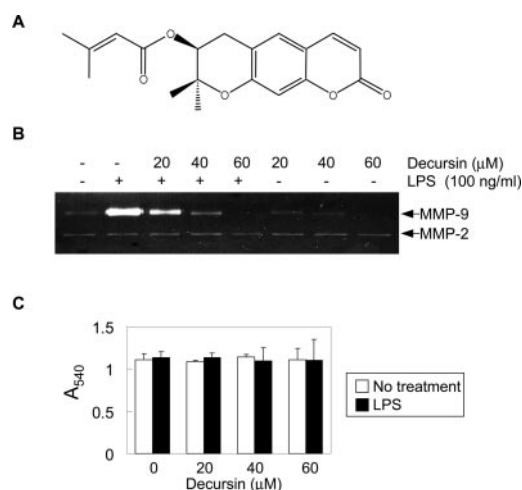
Herbal Drug	Specific Name	Family	Growing District	Product Number <sup>a</sup>	Suppressive Activity
Angelicae gigantis radix	<i>Angelica gigas</i>	Umbelliferae	Korea	CA01-031	+++
Cinnamomi ramulus	<i>Cinnamomum cassia</i>	Lauraceae	China	CA01-009	++
Trichosanthes semen	<i>Trichosanthes kirilowii</i>	Cucurbitaceae	China	CA02-018	–
Cnidii rhizoma	<i>Cnidium officinale</i>	Umbelliferae	Korea	CA03-071	+
Pinelliae tuber	<i>Pinellia ternata</i>	Araceae	China	CA04-015	+
Gardeniae fructus	<i>Gardenia jasminoides</i>	Rubiaceae	Korea	CA03-078	–
Aurantii nobilis pericarpium	<i>Citrus unshiu</i>	Rutaceae	Korea	CA03-068	–
Paeoniae radix alba	<i>Paeonia albiflora</i> Pallas	Paeoniaceae	Korea	CA02-034	–
Cyperii rhizoma	<i>Cyperus rotundus</i>	Cyperaceae	Korea	CA03-088	–
Liriodis tuber	<i>Liriope platyphylla</i>	Liliaceae	Korea	CA03-007	+
Ponciri fructur	<i>Poncirus trifoliata</i>	Rutaceae	China	CA02-094	–
Aurantii fructur	<i>Citrus aurantium</i>	Rutaceae	China	CA03-059	–
Atractylodis rhizoma	<i>Atractylodes japonica</i>	Compositae	China	CA03-069	–
Corydalis Tuber	<i>Corydalis ternata</i>	Papaveraceae	China	CA04-082	–

<sup>a</sup> From Plant Diversity Research Center (PDRC), Daejeon, Korea.

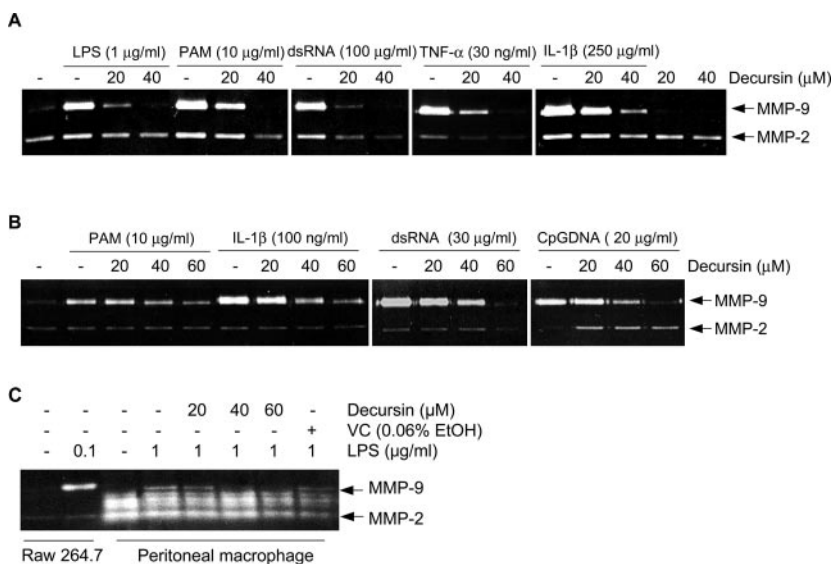




**Fig. 1.** The extracts from *A. gigas* and *C. cassia* block LPS-induced expression of MMP-9. RAW264.7 cells were incubated for 15 h in the presence of 10 to 30 µg/ml ethanol extracts from *A. gigas* radix (A), *C. cassia* ramulus (B), or *Trichosanthes kirilowii* semen (C) and then stimulated with 1 µg/ml LPS for 24 h. The culture supernatants were collected and subjected to gelatin zymogram as described under *Materials and Methods*. As a vehicle control, 0.1% ethanol was used.



**Fig. 2.** Decursin blocks MMP-9 expression in RAW264.7 cells stimulated with LPS without affecting cell viability. A, chemical structure of decursin. B, RAW264.7 cells were pretreated with 20, 40, or 60 µM decursin for 2 h before stimulation with 100 ng/ml LPS. The culture supernatants were collected 24 h after activation and subjected to gelatin zymogram. C, RAW264.7 cells were treated as described in B and cell viability was tested with MTT assay as described under *Materials and Methods*.



**Fig. 3.** Decursin blocks induction of MMP-9 in monocytic cell lines stimulated with various agents and in primary macrophages stimulated with LPS. THP-1 cells (A), RAW264.7 cells (B), and peritoneal macrophages (C) were pretreated with 20, 40, or 60 µM decursin for 2 h and activated with LPS, PAM, double-stranded RNA (poly I:C), TNF-α, IL-1β, or CpG 1826 DNA at indicated concentrations. As a vehicle control, 0.06% ethanol was used. The culture supernatants were then collected 24 h after the activation, and MMP-9 levels were analyzed using gelatin zymogram.

tested in these treatment conditions (Fig. 2C). These data indicate that decursin, which has been purified from the root of *A. gigas*, blocks MMP-9 induction in LPS-stimulated macrophages without affecting cytotoxicity. Decursin also blocked MMP-9 induction in LPS-treated human macrophage cell line THP-1 (Fig. 3A).

We then tested whether decursin blocks MMP-9 induction in cells activated with stimulants other than LPS. MMP-9 expression induced by PAM (a synthetic lipoprotein for TLR2 stimulation) was blocked by decursin in both THP-1 (Fig. 3A) and RAW264.7 cells (Fig. 3B) in a dose-dependent manner. MMP-9 expression induced by CpG DNA, the TLR9 ligand, was also blocked by decursin in RAW264.7 cells. Because stimulation of TLR4, TLR2, and TLR9 induce MyD88 mediated signaling, we stimulated the cells with IL-1β, which also induces MyD88-mediated signaling events. As expected, IL-1β-induced MMP-9 expression was blocked by decursin in both RAW264.7 cells and THP-1 cells. We then tested MyD88 independent stimulants such as double strand RNA (poly I:C, the TLR3 ligand) and TNF-α. Pretreatment of RAW264.7 and THP-1 cells with decursin blocked MMP-9 expression induced by these stimulants. These data indicate that the suppressive effect of decursin is not restricted to LPS stimulation but occurs in various pathways induced by cytokines and TLR ligands.

We then questioned whether the suppressive effect of decursin can occur in primary macrophages. Peritoneal macrophages, isolated from thioglycollate-injected mice, were stimulated with LPS in the presence of decursin. As shown in Fig. 3C, decursin blocked MMP-9 induction in these cells, indicating that the suppressive effect of decursin is not restricted to immortalized macrophage-like cells.

Next, we analyzed whether the suppressive effect of decursin is durable. For that purpose, we transiently exposed both RAW264.7 and THP-1 cells to decursin and then the decursin was removed from the culture medium by extensive washing. The suppressive effect of decursin was not detected in this condition (Supplemental Fig. 3).

We then tested whether decursin blocks induction of other inflammatory mediators, such as pro-inflammatory cytokines. Decursin blocked LPS-induced expression of MCP-1, IL-8, TNF-α, and IL-1β in a dose-dependent manner in

THP-1 cells (Fig. 4A). Decursin also blocked TNF- $\alpha$  expression in RAW264.7 cells activated with LPS (Fig. 4B). Furthermore, decursin blocked TNF- $\alpha$  production from peritoneal macrophages (Fig. 4C). These data demonstrate that decursin blocks LPS-induced cytokine expression in both immortalized and primary macrophages.

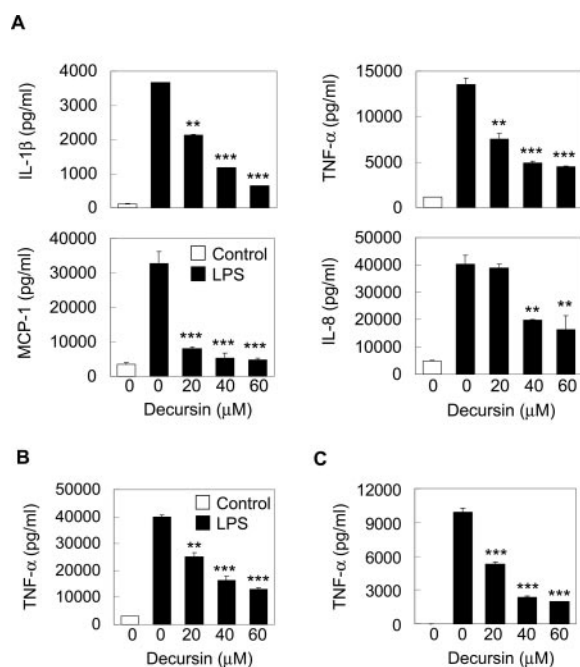
Next, we sought to determine whether decursin blocks activation of microglia, a form of macrophages resident in the central nervous system. Stimulation of BV-2 mouse microglia cells with LPS induced strong NO production. Decursin blocked the NO production in a dose-dependent manner (Fig. 5) without affecting cellular viability (data not shown). Inhibitory effects of decursin on the LPS-induced NO production were accompanied by the attenuation of inducible nitric oxide synthase (iNOS) induction (Fig. 5, inset). Taken together, our current data prove that decursin blocks inflammatory activation of macrophages with respect to the pro-inflammatory cytokine expression, NO production, and MMP-9 induction.

To find out whether decursin suppresses MMP-9 gene expression at transcriptional level, THP-1 cells were stimulated with LPS in the presence or absence of decursin and the relative amounts of MMP-9 mRNA were measured by real-time RT-PCR. As shown in Fig. 6A, treatment with decursin but not with the vehicle control blocked LPS induced up-regulation of MMP-9 mRNA levels. MMP-9 mRNA levels were also tested in RAW264.7 cells in different time points after LPS stimulation. Decursin completely blocked MMP-9 mRNA induction at 2 h after the LPS stimulation and approximately 70% at 4 h after the activation (Fig. 6B).

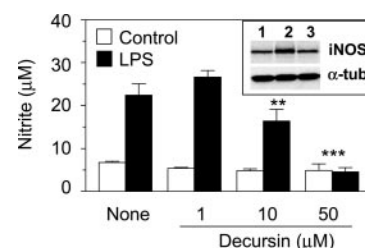
Because decursin blocked transcriptional activation of MMP-9 gene, it is likely that it blocks signaling events in-

involved in transcriptional activation of the MMP-9 gene. Expression of MMP-9 and cytokine genes requires NF- $\kappa$ B activation and its subsequent translocation into the nucleus. In resting cells, NF- $\kappa$ B/I $\kappa$ B complexes are present in the cytoplasm. Activation of cells under appropriate condition leads to phosphorylation and subsequent degradation of I $\kappa$ B. The free NF- $\kappa$ B then translocates into the nucleus to activate genes with NF- $\kappa$ B binding sites. Therefore, we tested whether decursin blocks NF- $\kappa$ B nuclear translocation. As shown in Fig. 7, LPS stimulation caused translocation of part, but not all, of the NF- $\kappa$ B from the cytoplasm into the nucleus within 60 min after the activation, whereas the presence of 80  $\mu$ M decursin blocked it (Fig. 7, compare A and B). Nuclear translocation of NF- $\kappa$ B was partially blocked in the presence of 20 and 40  $\mu$ M decursin and 80  $\mu$ M decursin blocked it completely (Fig. 7C). Decursin also blocked nuclear translocation of NF- $\kappa$ B in THP-1 cells activated with LPS (Supplemental Fig. 4). Decursin completely blocked phosphorylation and subsequent degradation of I $\kappa$ B in both RAW264.7 cells (Fig. 7, D-F) and in THP-1 cells (Supplemental Fig. 5). These data demonstrate that the suppression of I $\kappa$ B degradation and subsequent NF- $\kappa$ B nuclear translocation is responsible for the anti-inflammatory activities of decursin.

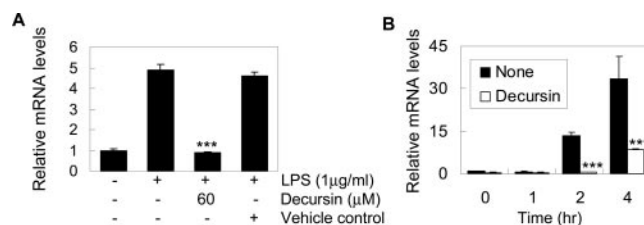
We then questioned whether the suppressive effect of decursin is restricted to NF- $\kappa$ B activation pathway or affects mitogen-activated protein kinase pathways as well. To test



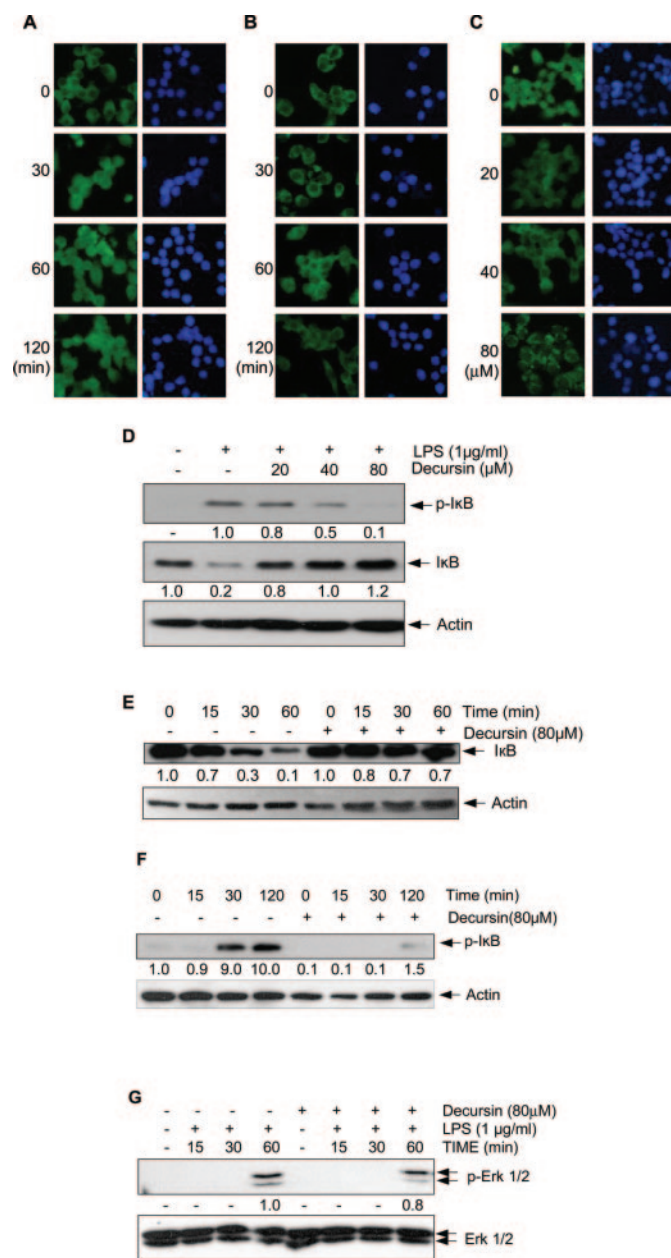
**Fig. 4.** Decursin blocks cytokine expression induced by LPS in monocytic cell lines. THP-1 cells (A), RAW264.7 cells (B), and peritoneal macrophages (C) were pretreated with 20, 40, or 60  $\mu$ M decursin for 2 h and activated with 1  $\mu$ g/ml LPS. Culture supernatants were collected in 24 h and the cytokine concentrations were measured using sandwich ELISA. Measurements were done in triplicate, and the error bars represent S.D. \*\*, significant difference from LPS treatment ( $P < 0.01$ ); \*\*\*,  $P < 0.001$ .



**Fig. 5.** Decursin blocks LPS-induced NO production in microglia cells. BV-2 microglial cells were treated with LPS (100 ng/ml) in the presence of 1 to 50  $\mu$ M decursin for 24 h, and then the production of nitric oxide was measured by Griess reaction. Values represent mean  $\pm$  S.D. \*, significant difference from LPS treatment ( $P < 0.05$ ); \*\*\*,  $P < 0.001$ . Inset, BV-2 cells were left untreated (lane 1) or treated with LPS (100 ng/ml) (lane 2) or LPS plus decursin (50  $\mu$ M) (lane 3) for 8 h, and then the expression of iNOS protein (top) was assessed by Western blot analysis. Detection of  $\alpha$ -tubulin was also carried out to confirm the equal loading of proteins (bottom).



**Fig. 6.** Decursin suppresses MMP-9 expression at transcriptional level. A, THP-1 cells, pretreated for 3 h with either 60  $\mu$ M decursin or 0.06% ethanol as a vehicle control, were stimulated with 1  $\mu$ g/ml LPS. One hour after the stimulation, total cellular RNAs were collected and subjected to real time RT-PCR analysis. B, RAW264.7 cells were treated with 80  $\mu$ M decursin overnight before stimulation with 1  $\mu$ g/ml LPS. Total cellular RNAs were collected at indicated times and used for real time RT-PCR analysis. Ct values from MMP-9 measurements were normalized with the values from corresponding GAPDH reactions. \*\*\*, significant difference from LPS treatment ( $P < 0.001$ ).



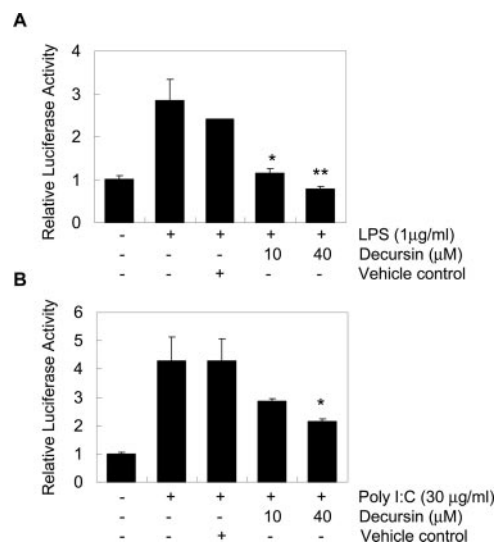
**Fig. 7.** Decursin blocks phosphorylation and degradation of IκB and activation of NF-κB. RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence (A) or presence (B) of 80 μM decursin that had been added 3 h before the activation. At the indicated times after LPS addition, subcellular location of NF-κB p65 subunit was tested using immunofluorescence assay. Note that nuclear translocation of p65 subunit is not complete but part of the cytoplasmic p65 translocated from the cytoplasm to the nucleus so that the distinction between the nucleus and the cytoplasm blurs 60 min after the activation. C, RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence or presence of 20, 40, or 80 μM decursin that had been added 3 h before the activation. Subcellular location of NF-κB p65 subunit was tested using immunofluorescence assay at 60 min after the activation. D, RAW264.7 cells were stimulated as in C. Total cell lysates obtained 1 h after the activation and the levels of IκB and its phosphorylated form in the cellular lysates were analyzed using Western blotting. E and F, RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence or presence of 80 μM decursin. The levels of IκB in the cellular lysates were analyzed using Western blotting. G, peritoneal macrophages were stimulated with 1 μg/ml LPS in the absence or presence of 80 μM decursin. The levels of Erk 1/2 and their phosphorylated forms were analyzed using Western blotting at indicated time points. Band intensities were measured by densitometer and normalized with band intensities of corresponding loading controls. The normalized quantification values are written below each band.

the activation of Erk, one of the three MAPKs, we used both peritoneal macrophages and RAW264.7 cells. As shown in Fig. 7G for the peritoneal macrophages, phosphorylation of Erk occurred 60 min after LPS treatment and the presence of 80 μM decursin failed to achieve a significant reduction in Erk phosphorylation. The same results were obtained in RAW264.7 cells, further confirming that decursin does not affect activation of Erk (Supplemental Fig. 6).

Because our data point out that decursin suppresses MMP-9 transcription through inhibition of NF-κB activation, we confirmed the suppressive effect of decursin on the NF-κB signaling pathway using NF-κB reporter assay. RAW264.7 cells were transiently transfected with plasmids, including a luciferase gene under the promoter containing four tandem repeats of NF-κB binding sites. As expected, decursin blocked the expression of the reporter gene after stimulation with either LPS or double-stranded RNA [poly(I:C)] (Fig. 8). These data further confirm that the decursin blocks signaling events leading to the activation of NF-κB.

## Discussion

Our present data provide the first evidence that decursin is an anti-inflammatory agent in macrophage functions. Decursin blocked various responses of macrophages to stimulants including TLR ligands and inflammatory cytokines. Although current data do not identify the molecular target, presence of decursin causes suppression of IκB degradation and subsequent NF-κB nuclear translocation. Because NF-κB is the key regulatory molecule in the transcriptional activation of the genes encoding MMP-9, proinflammatory cytokines, and iNOS (the enzyme responsible for the production of NO), treatment with decursin will lead to the transcriptional suppression of these genes. In the case of MMP-9, we confirmed that decursin blocked induction of MMP-9 mRNA levels in macrophages activated with LPS (Fig. 6).



**Fig. 8.** Decursin blocked NF-κB activation in luciferase reporter assay. RAW264.7 cells were transiently transfected with plasmids containing firefly luciferase reporter gene under a promoter containing four tandem repeats of NF-κB binding site and *R. reniformis* luciferase expression construct as an internal control. The transfected cells were then pre-treated with 10 or 40 μM decursin for 2 h and activated with LPS (A), or poly I:C (B) for 6 h at indicated concentrations before measuring the luciferase activities. As a vehicle control, 0.04% ethanol was used. \*, significant difference from LPS treatment ( $P < 0.05$ ); \*\*,  $P < 0.01$ .



Based on the present findings, decursin must be added to what is now a very long list of compounds, some natural and others synthetic, that inhibit signaling via the NF- $\kappa$ B pathway. Like decursin, a large number of these compounds are electrophiles that are characterized by the presence of an olefinic linkage conjugated to the C=O group of a ketone, ester, or lactone. Two of these conjugated systems are present in the molecular structure of decursin. Why compounds in this general category tend to inhibit NF- $\kappa$ B-dependent signaling remains to be determined.

Decursin needs to be present continuously for the inhibition of LPS-induced expression of MMP-9 (Supplemental Fig. 3). This indicates that the effect of decursin does not occur through a covalent modification of its molecular targets. It is possible that decursin is an inhibitor of an enzyme, such as kinase, involved in LPS induced NF- $\kappa$ B activation. It is also possible that decursin may interfere with the interaction between signaling molecules in NF- $\kappa$ B activation pathway. The inhibitory effect of decursin seems to be specific to NF- $\kappa$ B signaling pathway, because addition of 80  $\mu$ M decursin, which was sufficient to suppress NF- $\kappa$ B activation, failed to block phosphorylation of Erk.

The suppressive effect of decursin is not restricted to cultured cell lines; it can occur in primary macrophages. Thus, it is expected that decursin will have similar effect in vivo, unless decursin is rapidly converted into other inactive compounds via metabolic enzymes or masked by some serum components, and so forth.

Decursin has been studied as a chemical with various biological activities including anticancer, antioxidant, antiplatelet aggregation, and antibacterial activities. The antitumor activity of decursin was reported by Lee et al. (2003); decursin not only increased the survival time of mice injected with the Sarcoma-180 ascitic tumor but also reduced the tumor volumes and weights. The underlying mechanism of decursin's antitumor activity has been investigated by Yim et al. (2005), who reported that decursin treatment induced G<sub>1</sub> or G<sub>2</sub>-M arrest in human prostate carcinoma cells. Furthermore, decursin caused apoptosis in the cancer cells via both caspase-dependent and -independent mechanisms.

Our data might provide additional mechanism responsible for the antitumor activity of decursin. It is well noted that inflammation is linked to cancer development (Clevers, 2004; Philip et al., 2004; Schwartzburd, 2004; Crowe, 2005) and activation of MMPs is involved in tumor metastasis (Klein et al., 2004; Mannello et al., 2005; Vihinen et al., 2005). The suppression of cytokine/MMP-9 expression and subsequent inflammation by decursin may contribute to its antitumor effect. Whether decursin can block inflammatory activities associated with oncogenesis and metastasis remains to be investigated.

In addition to the activities described above, decursin exhibited significant protective activity against glutamate-induced neurotoxicity when added to primary cultures of rat cortical cells at concentrations ranging from 0.1 to 10  $\mu$ M (Kang et al., 2005). Decursin may exert neuroprotective effects because of its inhibitory effects on neurotoxic microglial activation as determined in Fig. 5.

Our data added one more effect of decursin, anti-inflammatory effects on macrophages, to the growing number of its biological effects. Further investigation is required to

find out the molecular target(s) of this compound in NF- $\kappa$ B pathway and to find out whether this compound can suppress other inflammatory reactions mediated by macrophages.

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