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NF-κB-dependent Regulation of Matrix Metalloproteinase-9 Gene Expression by Lipopolysaccharide in a Macrophage Cell Line RAW 264.7

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Matrix metalloproteinase-9 (MMP-9) plays a pivotal role in the turnover of extracellular matrix (ECM) and in the migration of normal and tumor cells in response to normal physiologic and numerous pathologic conditions. Here, we show that the transcription of the MMP-9 gene is induced by lipopolysaccharide (LPS) stimulation in cells of a macrophage lineage (RAW 264.7 cells). We provide evidence that the NF-κB binding site of the MMP-9 gene contributes to its expression in the LPS-signaling pathway, since mutation of NF-κB binding site of MMP-9 promoter leads to a dramatic reduction in MMP-9 promoter activation. In addition, the degradation of IκBα, and the presences of myeloid differentiation protein (MyD88) and tumor necrosis factor receptor-associated kinase 6 (TRAF6) were found to be required for LPS-activated MMP-9 expression. Chromatin immunoprecipitation (ChIP) assays showed that functional interaction between NF-κB and the MMP-9 promoter element is necessary for LPS-activated MMP-9 induction in RAW 264.7 cells. In conclusion, our observations demonstrate that NF-κB contributes to LPS-induced MMP-9 gene expression in a mouse macrophage cell line.

Keywords: LPS, Macrophages, MMP-9, NF-κB

Introduction

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are well-known mediators of cell migration, invasion, proliferation and angiogenesis in response to normal physiologic and numerous pathologic conditions (van den Steen *et al*., 2002; Yoon *et al*., 2003). MMPs are produced by inflammatory cells like macrophages, lymphocytes, neutrophils, and eosinophils (Visse and Nagasse, 2003), and several investigators have shown that MMPs cause cell migration in response to multiple mediators, such as, growth factors, cytokines, and bacterial products like LPS (Bond *et al*., 1998; Opdenakker *et al*., 2001; Dubois *et al*., 2002). In particular, matrix metalloproteinase-9 (MMP-9), a secreted type IV collagenase, has been implicated in several inflammatory diseases, e.g., inflammations of the pulmonary tract, gastrointestinal tract, and joints (Hoshino *et al*., 1998; van den Steen *et al*., 2002). Moreover, MMP-9 can degrade extracellular matrix components like collagens and elastins, and when activated, plays a central role in cell migration (Galis *et al*., 1995; Klein *et al*., 2004).

MMP-9 expression is enhanced by multiple mediators, which include PMA, TNF-α, and bacterial products like LPS and CpG-ODN (van den Steen *et al*., 2002; Holvoet *et al*., 2003; Islam and Nabi, 2003; Rhee *et al*., 2007), the latter of which are synthetic oligodeoxynucleotides that contain unmethylated CpG dinucleotides, which stimulate MMP-9 gene transcription. Binding sites for the transcription factors AP-1, NF-κB, Ets, and Sp1 have been identified in the proximal promoter of the MMP-9 gene. In general, the proximal AP-1 site is required for MMP-9 expression (Sato and Seiki, 1993), whereas the NF-κB and Sp1 sites are additionally required for the induction of the MMP-9 gene in response to PMA and TNF-α (van den Steen *et al*., 2002). Moreover, it has been reported that CpG-ODN has a chemotactic effect on macrophages and that the activations of p38 MAP kinase, ERKs, and PI3K play important roles in chemotactic migration (Baek *et al*., 2001). In our previous

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study (Rhee *et al*., 2006), CpG-ODN was identified as a potential mediator of MMP-9 gene expression in RAW 264.7 cells *via* the activation of NF-κB transcription factor. Moreover, MMP-9 activation in response to CpG-ODN was also found to be an important mediator of macrophage chemotactic activity. Furthermore, Woo *et al*. showed that the induction of MMP-9 gene expression by LPS is mediated by a mitochondria-derived ROS-p38 kinase cascade that leads to the stimulation of AP-1 in an NF-κB-independent manner (Woo *et al*., 2004).

Here, we show the involvement of NF-κB activation in LPS-induced MMP-9 gene expression in RAW 264.7 cells. Experimental evidence showed that NF-κB activation is a key transcription factor for MMP-9 gene expression, and that NF-κB-dependent MMP-9 expression plays an important role in LPS-induced cell migration.

Materials and Methods

Cell culture and reagents. RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA; CRL 2278). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10 mg/ml streptomycin. Viability was assayed by trypan blue dye exclusion, and was typically greater than 95%. Cultures were maintained until passage 20, and then discarded.

Pyrrolidine dithiocarbamate (PDTC) was purchased from A. G. Scientific Inc (San Diego, CA). *Escherischia coli* LPS (Sigma) was suspended in sterile water and added to cell culture to the desired concentrations. Phosphorothioate backbone-modified oligodeoxy-nucleotides were purchased from GenoTech (Daejon, Korea). CpG-ODN 1826 consisted of 20 bases containing two CpG modifs (underlined): TCCATGACGTTCCTGACGTT. Tissue inhibitor of metalloproteinase-1 (TIMP-1) was obtained from R&D Systems, Inc. (Minneapolis, MN). The expression vector for mutant IκBα protein (IκBα Supper repressor, IκBαSR), which cannot be phosphorylated at serines 32 and 36, was used as previously described (Kim *et al*., 2003). The following plasmids expressing dominant negative mutant proteins were used for transfection experiments: ΔMyD88 (Burns *et al*., 1998), ΔTRAF2 (Song *et al*., 1997), and ΔTRAF6 (Cao *et al*., 1996).

***In vitro* migration assays.** Transwell migration assays were performed using a modification of the 96-well microchemotaxis assay previously described (Bacon *et al*, 1988; Rhee *et al*., 2007). In brief, RAW 264.7 cells were loaded into top chambers (Corning Costar) and LPS into bottom chambers and incubated for 6 h at 37°C in 0.5% bovine serum albumin/DMEM medium in a humidified 5% CO_2 incubator. In some experiments, cells were pre-treated with TIMP-1 to inhibit MMP-9 activity or PDTC to inhibit NF-κB. Cells that had migrated to lower filter surfaces were fixed with 4% paraform-aldehyde in PBS for 10 min at room temperature (RT) and stained with hematoxylin and eosin Y. Cell migration was defined as the number of cells that had migrated to lower filter surfaces.

Reverse-transcription PCR analysis. After treating cells with LPS (100 ng/ml), TIMP-1 (50 nM), and/or PDTC (200 mM) for the indicated times, total RNA was extracted using total RNA isolation Kits (Active Motif). RT-PCR was performed as described previously (Rhee *et al*., 2007).

Construction of luciferase reporter plasmid. MMP-9-Luc reporter construct containing bp -664 to +75 of the MMP-9 gene immediately upstream of firefly luciferase cDNA has been described (Rhee *et al*., 2007). To introduce a site-specific mutation in the NF-κB binding site, we abrogated the transcription factor recognition site and replaced with a non-functional sequence using the two-step PCR mutagenesis method (Lee *et al*., 2005). This method uses 5'-primer, MMP-9(-664) 5'-GGATAGTGCTAGCCTG AG-3' and 3'-primer, MMP-9(+75) 5'-CTCGAGCTGCAGCCGAA AGCCAG-3', along with primers that encode the following sequences in a sense or antisense orientation; mNF-κB, 5'-GGatcgATTCCCCAAATCCTGCCTCAA-3'. Mutated sites are indicated by lowercase letters. We cloned the promoter sequence, including the mutation, into pGL3-Basic vector, to produce the reporter construct pMMP-9(mNF-κB)-*Luc*.

Transfection and luciferase assays. For transient transfection assays, RAW 264.7 cells were placed in 12-well plates at 1×10^5 cells/well. FuGene 6 Transfection Reagent (Roche) was used to introduce plasmid constructs into cells as previously described (Kwon *et al*., 2003). For comparisons between constructs, we confirmed equivalent transfection efficiency by cotransfecting the promoterless *Renilla* luciferase vector pRL-null (Promega) as an internal control (Jing *et al*., 2004; Lee *et al*., 2004). After transfection, cells were placed in complete medium for 24 h prior to being treated with LPS (100 ng/ml) for 12 h. Luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Tuner Designs) according to the manufacturer's instructions.

Measurement of MMP-9 activity by gelatin zymography. A gelatin zymography assay system was employed in order to identify MMP-9 activity, as described previously (Rhee *et al*., 2007). Protein samples of culture medium were loaded and electrophoresed in 7.5% SDS-polyacrylamide gels containing 200 μg/ml gelatin. Gels were washed with 2.5% Triton X-100 for 1 h and incubated at 37°C for 20 h in a reaction buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 10 mM $CaCl_2$. The gels were then stained with Coomassie Brilliant Blue R-250 (0.2%) in 40% methanol and 10% acetic acid.

Chromatin Immunoprecipitation assay. Chromatin was prepared from RAW 264.7 cells treated with LPS or CpG-ODN 1826 for 30 min and chromatin immunoprecipitation (ChIP) assays were performed as described previously (Lee *et al*., 2006). DNA immunoprecipitated by NF-κB p65 antibody was analyzed by PCR using promoter-specific primer pairs for the NF-κB binding site of MMP-9 promoter. The following primer set was used: 5'-CTTTAA ACAGAAGAGGAAGGATAGTGC-3' and 5'-CCTGATAGAGTC TTTGACTCAGCTTC-3'. DNA purified from sonicated nuclear lysates was directly analyzed by PCR using the same primer set, which was used as an input control.

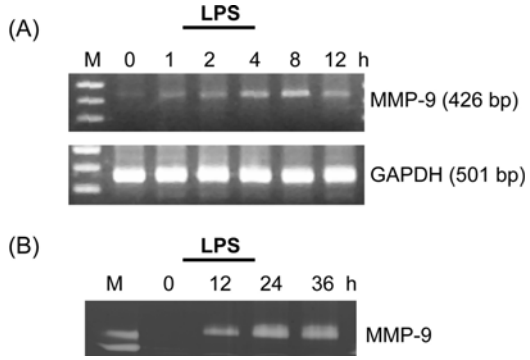

Fig. 1. Time course of LPS-induced MMP-9 expression in RAW 264.7 cells. (A) RAW 264.7 cells were treated with LPS (100 ng/ml) for the indicated times. MMP-9 mRNA expression (upper panel, 426 bp) was analyzed by RT-PCR. GAPDH expression was used as a control (lower panel, 501 bp). M, DNA standard marker. (B) RAW 264.7 cells were incubated with LPS (100 ng/ml) for the indicated times. Culture media protein samples were loaded and electrophoresed in 7.5% SDS-polyacrylamide gels containing 200 μg/ml gelatin. MMP-9 activities were determined by zymography, as described in Materials and Methods.

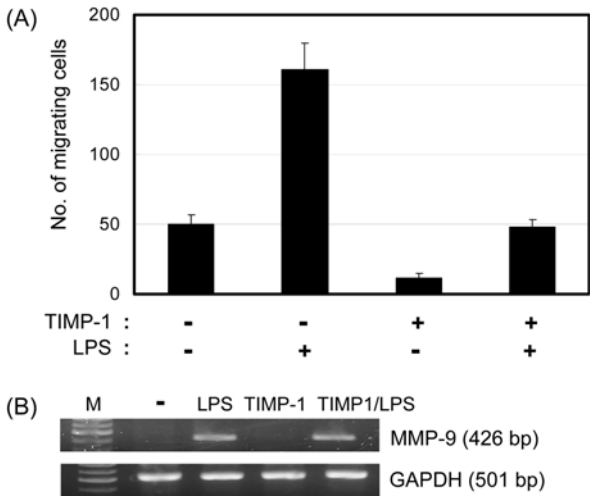

Fig. 2. MMP-9-dependent migration of LPS stimulated RAW 264.7 cells. (A) Effect of LPS or TIMP-1 on RAW 264.7 migration. 5×10^4 cells were preincubated with 50 nM TIMP-1 for 1 h, and then loaded into the upper wells of transwell chambers which contained LPS (100 ng/ml) in lower chambers. After incubation in a humidified 5% CO_2 incubator for 6 h, hematoxylin and eosin Y stained cells on lower filter surfaces were examined under a microscope. Cell migration was quantified by counting cells that had migrated across filters. (B) Effect of TIMP-1 on MMP-9 expression in LPS-treated RAW 264.7 cells. RAW 264.7 cells were preincubated with 50 nM TIMP-1 for 1 h, and then stimulated with 100 ng/ml LPS for 8 h. MMP-9 mRNA expressions (upper panel, 426 bp) were analyzed as described in Fig. 1A.

Results and Discussion

LPS induces MMP-9 expression. LPS has been reported to increase MMP-9 production via a MAPK pathway, particularly ERK and p38 pathways, in monocytes (Lai *et al.*, 2003), astrocytes (Lee *et al.*, 2003), and neutrophils (Underwood *et al.*, 2000). Here, we examined the induction of endogenous MMP-9 gene expression in LPS-treated RAW 264.7 cells using reverse-transcription PCR assays. As shown in Fig. 1A, the MMP-9 gene was endogenously expressed in RAW 264.7 cells, and LPS enhanced its expression in a time-dependent manner. We also examined the MMP-9 activity released from LPS-treated cells using gelatin zymography assays. After stimulating RAW 264.7 cells with LPS, biologically active MMP-9 in cell culture supernatant increased in a time-dependent manner (Fig. 1B). These results are in agreement with previous report (Woo *et al.*, 2004) and suggest that LPS-stimulated RAW 264.7 cells not only have higher level of MMP-9 gene expression but that they also release biologically active MMP-9 protein into medium.

MMP-9-dependent migration of LPS-treated RAW 264.7 cells. MMPs cause cell migration in response to multiple mediators (like LPS) in inflammatory cells (Bond *et al.*, 1998; Opdenakker *et al.*, 2001; Dubois *et al.*, 2002). As we found that LPS enhanced MMP-9 mRNA expression and enzymatic activity in RAW 264.7 cells, we next examined whether LPS-induced cell migration is regulated by MMP-9 activity. As shown in Fig. 2A, RAW 264.7 cell migration increased about three-fold in response to LPS in transwell migration assays. To evaluate the effect of MMP activity on RAW 264.7 cell migration, we used TIMP-1 to inhibit MMP-9 activity. When cells were pretreated with TIMP-1, basal cell migration was reduced by about 70%. Furthermore, the number of cells migrating in response to LPS was significantly reduced by TIMP-1, as shown in Fig. 2A. In contrast, MMP-9 mRNA basal levels were unchanged by treating control cells with TIMP-1 (Fig. 2B). These results imply that LPS-induced RAW 264.7 cell migration is controlled by MMP-9 activity.

NF-κB activation is required for LPS-induced MMP-9 promoter activation. To determine if NF-κB p65 is required for the transcriptional activation of the MMP-9 gene, we co-transfected expression plasmid encoding NF-κB p65 into RAW 264.7 cells with a MMP-9 promoter-reporter construct and then checked luciferase activity. Co-transfection with NF-κB p65 expression vector at 100 ng/ml conferred about two-fold activation over this (empty expression vector, pTL-1) basal transcriptional level. In addition, we determined whether NF-κB p65 is necessary for the LPS-dependent activation of MMP-9 promoter. As shown in Fig. 3A, treatment of RAW 264.7 cells with LPS resulted in around a five-fold increase in MMP-9 promoter activity. Moreover, LPS-induced transactivation of MMP-9 promoter was dramatically increased when RAW 264.7 cells were co-transfected with NF-κB p65 expression vector (Fig. 3A). To confirm these observations, we examined the involvement of NF-κB in the transcriptional upregulation

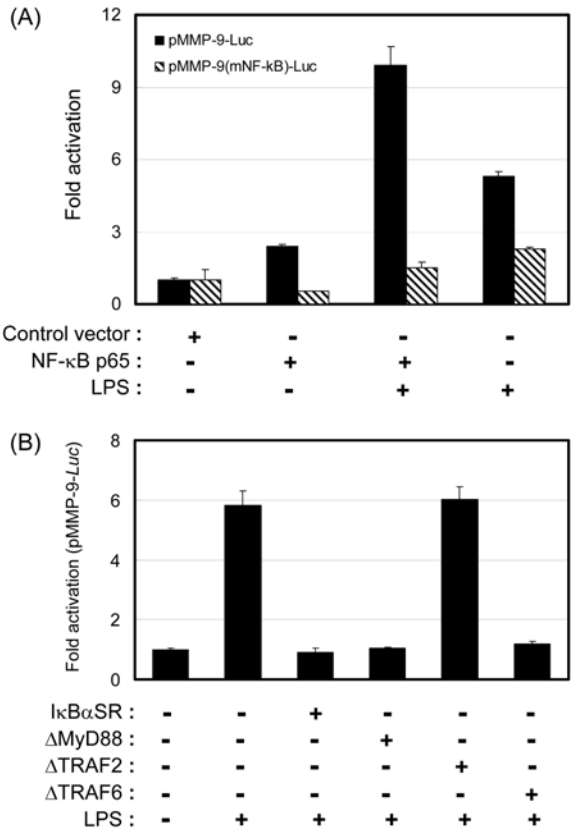

Fig. 3. Effect of NF-κB activation on MMP-9 transcriptional activation in response to LPS. (A) RAW 264.7 cells were transfected with reporter constructs and plasmids constructs and plasmids expressing NF-κB p65 for 12 h, and then stimulated with 100 ng/ml of LPS for 12 h. Cultures were harvested and assayed for luciferase activity. (B) LPS-induced MMP-9 promoter activity in RAW 264.7 cells was inhibited by dominant negative mutants of MyD88 or TRAF6. RAW 264.7 cells were transiently cotransfected with MMP-9 promoter-reporter construct plus control vector, or IκBαSR, and dominant negative mutants of MyD88, TRAF2, or TRAF6 for 24 h. Cells were then treated with 100 ng/ml LPS for 12 h. Cultures were harvested and assayed for luciferase activity. Luciferase activity was measured in relative light units normalized versus Renilla activity. Results represent fold activations versus the control vector.

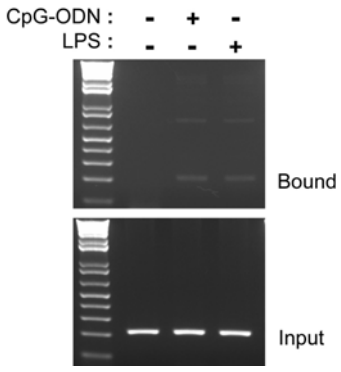

Fig. 4. Direct binding of NF-κB to MMP-9 promoter in response to LPS. Formaldehyde cross-linked chromatin fragments isolated from 100 ng/ml LPS- or 3 mM CpG-ODN 1826-treated RAW 264.7 cells were immunoprecipitated with NF-κB p65 antibody. Immunoprecipitates were analyzed by PCR for the presence of the MMP-9 gene promoter sequence using the primer pair described in Materials and Methods (Bound). DNA purified from sonicated chromatin was directly analyzed by PCR using the primer set used as an input control (Input).

of MMP-9 promoter by analyzing the NF-κB binding site-specific mutant construct after the ectopic expression of NF-κB p65. The mutation in the NF-κB binding site of pMMP-9-*Luc* abolished the enhancement of promoter activity in response to NF-κB p65, and the mutation in the NF-κB site also substantially reduced dramatic responses to the ectopic expression of NF-κB p65 in LPS-stimulated cells (Fig. 3A).

Generally, LPS stimulation results in the degradation of IκB and in the subsequent release and translocation of NF-κB to the nucleus, where it activates various genes. In the present study, we examined whether MMP-9 promoter activation is modulated by LPS signaling-dependent IκBα degradation. When RAW 264.7 cells were transfected with mutant IκBα (IκBα Super Repressor, IκBαSR), which cannot be phosphorylated at serines 32 and 36, the induction of MMP-9 promoter activity by LPS was abolished (Fig. 3B). Taken together, these findings demonstrate that the NF-κB signaling pathway implicated in response to LPS is required for the induction of MMP-9 gene expression in RAW 264.7 cells.

Signaling by LPS through TLR4 requires the participation of the adaptor proteins MyD88 and TRAF6, and results in the activation of the transcription factor NF-κB (Zhang *et al*., 1999). To determine whether MyD88 is involved in MMP-9 promoter activation, along with the promoter we co-transfected an expression plasmid encoding mutant MyD88 (ΔMyD88) into RAW 264.7 cells. As expected, ΔMyD88 co-expression inhibited LPS-mediated MMP-9 promoter activation (Fig. 3B). We then examined requirements for TRAF2 and TRAF6 in LPS-induced MMP-9 promoter activation. As shown in Fig. 3B, ΔTRAF6 but not ΔTRAF2 significantly reduced the LPS-induced activation of MMP-9 promoter. Thus, indicating that the signal transduction molecules MyD88 and TRAF6 in the TLR/IL-1R signaling pathway are required for MMP-9 gene expression induction in mouse macrophages stimulated by LPS.

Direct interaction between NF-κB and MMP-9 promoter. To determine whether NF-κB directly binds to MMP-9 promoter *in vivo* after its signal-induced nuclear accumulation in LPS-stimulated cells, we used ChIP assays (Fig. 4). To confirm the interaction between NF-κB p65 and genomic DNA complex, formaldehyde cross-linked chromatin fragments were isolated from RAW 264.7 cells, immunoprecipitated with NF-κB p65 antibody, and analyzed by PCR for the presence of a specific DNA sequence corresponding to the NF-κB binding site -540 to -531 in mouse MMP-9 promoter.

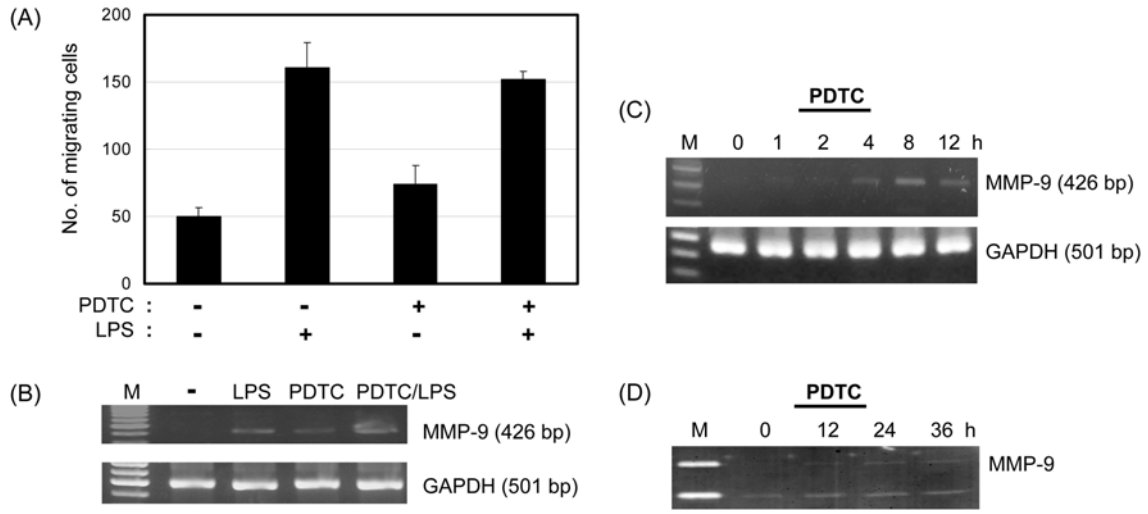

Fig. 5. PDTC stimulates MMP-9 expression in RAW 264.7 cells. (A) Effect of LPS and PDTC on RAW 264.7 cell migration. 5×10^4 cells were preincubated with 200 mM PDTC for 1h, and then loaded into the upper wells of transwell chambers containing LPS (100 ng/ml) in lower chambers. Cell migration was determined as described in Fig. 2A. (B and C) Effect of PDTC on MMP-9 gene expression. RAW 264.7 cells were preincubated with 200 mM PDTC for 1h, and then stimulated with 100 ng/ml LPS for 8 h (B). RAW 264.7 cells were treated with PDTC (200 mM) for the indicated times, and then total RNA was extracted (C). MMP-9 mRNA expression (upper panel, 426 bp) was analyzed as described in Fig. 1A. (D) RAW 264.7 cells were incubated with PDTC (200 mM) for the indicated times. Protein samples of culture media were loaded and electrophoresed in 7.5% SDS-polyacrylamide gels containing 200 mg/ml gelatin. MMP-9 activity was determined by zymography as described in Materials and Methods.

As expected, the NF-κB binding region in MMP-9 promoter was immunoprecipitated by anti-NF-κB p65 antibody when cells were treated with LPS (Fig. 4). As previously shown (Rhee *et al*., 2007), the NF-κB p65 and NF-κB binding site complex was also found to be formed by ChIP assay when RAW 264.7 cells were treated with CpG-ODN 1826. This result provides clear evidence that NF-κB directly binds to the NF-κB binding site in MMP-9 promoter region in LPS activated RAW 264.7 cells.

Effects of PDTC on MMP-9 gene expression and the release of biologically active MMP-9. We previously reported that NF-κB contributes to LPS-induced cytokine gene expression in RAW 264.7 cells (Kim *et al*., 2003; Kwon *et al*., 2003). Here, we also show that NF-κB is essential for MMP-9 gene expression and for the release of biologically active MMP-9 in response to LPS. To confirm the involvement of NF-κB activity in LPS-induced cell migration and MMP-9 gene expression, we used PDTC to specifically inhibit NF-κB activation. Interestingly, even after being pretreated with PDTC, RAW 264.7 cells showed enhanced migration in response to LPS. Furthermore, PDTC itself was able to enhance cell migration in the absence of LPS stimulation (Fig. 5A). To evaluate the effect of PDTC on LPS-induced MMP-9 gene expression, RT-PCR assays were performed on PDTC-pretreated cells. As shown in Fig. 5B, PDTC pretreatment was not effective at inhibiting LPS-responsive MMP-9 gene expression. Furthermore, PDTC itself was able to induce MMP-9 gene expression in the absence of LPS stimulation in a time-dependent manner (Fig. 5C). We also found using gelatin zymography assays that PDTC induced MMP-9 activity in RAW 264.7 cells. After stimulating RAW 264.7 cells with PDTC, the biologically active MMP-9 in cell culture supernatant increased in a time-dependent manner (Fig. 5D), indicating that PDTC is able to induce MMP-9 expression in RAW 264.7 cells. Previously, we reported that the LPS-induced degradation of IκBα and translocation of NF-κB p65 were completely abolished by pretreating RAW 264.7 cells with PDTC, and that NF-κB p65 is not activated in cells treated with PDTC alone (Sohn *et al*., 2005). Therefore, we propose that PDTC (a typical NF-κB inhibitor) activates AP-1 via the SAPK/JNK pathway, and that this activated AP-1 binds to the AP-1 sites in the promoter region to cause the expressions of proinflammatory cytokine genes in an NF-κB-independent manner (Sohn *et al*., 2005). Taken together, these results imply that PDTC alone is able to induce MMP-9 gene expression in an NF-κB-independent manner in RAW 264.7 cells. Therefore, we believe that MMP-9 activation in response to LPS in the presence of PDTC does not mean that NF-κB is not involved in these phenomena.

Cells of the monocyte/macrophage lineage can be induced to express MMP-9, e.g., LPS stimulates monocytic cells to induce MMP-9 (Lai *et al*., 2003). MMP-9 is regulated primarily at the level of gene transcription, and AP-1 and NF-κB are transcription factors that are critically required for the expression of MMP-9 in response to PMA or TNF-α (Van den Steen *et al*., 2002). Woo *et al*., showed that the induction of MMP-9 gene expression by LPS is mediated by a mitochondria-derived ROS-p38 kinase cascade that leads to the stimulation of AP-1 in RAW 264.7 cells (Woo *et al*.,

2004), and that PDTC (a NF-κB inhibitor), does not inhibit MMP-9 expression in response to LPS *via* suppressing NF-κB activation. In contrast to these results, we previously found that the typical NF-κB inhibitor PDTC activates AP-1 via a SAPK/JNK pathway in RAW 264.7 cells, and that the activated AP-1 binds to AP-1 sites in the promoter region to induce the expressions of proinflammatory cytokine genes such as TNF-α, MIP-2 and GM-CSF in an NF-κB-independent manner (Sohn *et al*., 2005), and here, we show that MMP-9 gene expression in the presence of PDTC offers another example. Furthermore, we provide evidence that the NF-κB binding site contributes to MMP-9 gene expression in the LPS-signaling pathway, since mutation of the NF-κB binding site in MMP-9 promoter was found to lead to a dramatic reduction in the MMP-9 promoter activation induced by LPS treatment and/or by the ectopic expression of NF-κB p65. Moreover, ChIP assays demonstrated that the NF-κB binding site of MMP-9 promoter can form a complex with LPS-activated NF-κB in RAW 264.7 cells. Thus, our experimental results demonstrate that NF-κB contributes to LPS-induced mouse MMP-9 gene expression in the RAW 264.7 macrophage cell line.

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