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Toll-like receptor 9 dependent activation of MAPK and NF-κB is required for the CpG ODN-induced matrix metalloproteinase-9 expression

Eun-Jung Lim[1,3]*, Sun-Hye Lee[1,3]*, Jin-Gu Lee[1,3], Jae-Ryong Kim[1,3], Sung-Su Yun[2], Suk-Hwan Baek[1,3] and ChuHee Lee[1,3,4]

[1]Department of Biochemistry and Molecular Biology
[2]Department of Surgery
[3]Aging-Associated Vascular Disease Research Center
College of Medicine, Yeungnam University
Daegu 705-717, Korea
[4]Corresponding author: Tel, 82-53-620-4522;
Fax, 82-53-654-6651; E-mail, chlee2@ynu.ac.kr
*These authors contributed equally to this work.

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Abbreviations: CpG ODN, CpG oligodeoxynucleotide; MMP, matrix metalloproteinase; TLR9, toll-like receptor 9

Abstract

Unmethylated CpG oligodeoxynucleotides (CpG ODNs) activate immune cells to produce immune mediators. This study demonstrates that in murine macrophage RAW 264.7 cells, CpG ODN-mediated matrix metalloproteinase-9 (MMP-9) expression is regulated at transcriptional level and requires *de novo* protein synthesis. Inhibition of ERK and p38 MAPK, but not JNK, results in significant decrease of CpG ODN-induced MMP-9 expression. We found that endosomal maturation inhibitors, chloroquine and bafilomycin A, block CpG ODN-induced ERK and p38 MAPK activation and the subsequent MMP-9 expression. We also observed that CpG ODN induces NF-κB activation and NF-κB is a downstream target of p38 MAPK. Taken together, our data demonstrate that CpG ODN triggers MMP-9 expression via TLR-9 dependent ERK and p38 MAPK activation followed by NF-κB activation.

Keywords: CpG-oligonucleotide; extracellular signal-regulated MAP kinases; matrix metalloproteinase 9; NF- kappa B; p38 mitogen-activated protein kinases; Toll-like receptor 9

Introduction

Pathogen-derived structures activating the innate immune system include: cell wall products such as LPS and peptidoglycan, nucleic acids such as dsRNA, viral RNAs, and bacterial DNA (Janeway and Medzhitov, 2002). Bacterial DNA has been shown to directly target NK cells, B cells, macrophages, and dendritic cells and plays an important role in the innate immune system (Yamamoto *et al.*, 1992; Krieg *et al.*, 1995; Stacey *et al.*, 1996; Sparwasser *et al.*, 1998). Immunostimulatory activity of bacterial DNA depends on unmethylated CpG dinucleotides in particular base contexts ("CpG motif") (Klinman *et al.*, 1996; Krieg, 2002). Synthetic oligodeoxynucleotides containing CpG motifs (CpG oligodeoxynucleotides, CpG ODNs) mimic bacterial DNA immunostimulatory activity (Yi *et al.*, 1998).

CpG ODNs are recognized by a Toll-like receptor (TLR) 9 (Hemmi *et al.*, 2000), which is expressed in the endoplasmic reticulum and transposes to DNA-containing endosomes (Latz *et al.*, 2004). Cellular recognition of bacterial DNA occurs after nonsequence-specific receptor-mediated uptake and endosomal acidification (Hacker *et al.*, 1998, Macfarlane and Manzel, 1998). The nature of the receptor(s) mediating the uptake of exogenous DNA remains uncertain. Cytoplasmic domains allow TLRs to use signaling molecules used by the interleukin 1 receptors (IL-1Rs): these include MyD88, IL-1R-associated protein kinase and tumor necrosis factor receptor-activated factor 6. Signal transduction pathways activated by TLRs induce the transcription of a series of cytokine/chemokine genes that are involved in the initiation or regulation of the inflammatory response. However, the recent progress in our understanding of TLR functions is accumulating that the signaling pathways associated with each TLR are not identical and may result in different biological responses.

Matrix metalloproteinase (MMP) is capable of basement membrane degradation *in vivo*. MMP-9 expression normally only occurs in trophoblasts, osteoclasts, and leukocytes and their precursors and is involved in migration and infiltration of immune and inflammatory cells (Borregaard *et al.*, 1995; Harvey *et al.*, 1995; Witty *et al.*, 1996). Transcription of MMP-9 can be readily induced by a multitude of

agents including growth factors, cytokines, adhesion molecules, and other factors which induce cell shape alteration (Dong *et al.*, 2001; Martin *et al.*, 2001). Recently, we reported that CpG ODN is an inducer of MMP-9 through TLR-9 and Akt pathway (Lim *et al.*, 2006).

MAPK cascades are evolutionarily well conserved in all eukaryotic cells. These kinases play an important role in linking a variety of extracellular signals to cellular events such as growth, differentiation, apoptosis and inflammatory response (Chang and Karin, 2001). The activation of MAPK cascades, which in turn activates NF-κB, has been well characterized in cells of the mammalian immune system (Hacker *et al.*, 2002). NF-κB plays a central role in inflammation by regulating expression of genes that encode pro- inflammatory cytokines, chemokines and inducible enzymes such as iNOS in mammalian immune cells (Hanada and Yoshimura, 2002). Induction of cell signaling pathways including MAPK and NF-κB in response to CpG ODN has been reported in various cells. For example, CpG ODN-stimulated plasmacytoid dendritic cells (PDCs), NK cells, and B cells produce Th1-like proinflammatory cytokines, interferons, and chemokines (Krieg, 2002).

In this study, we demonstrated that CpG ODN elicits MMP-9 release from murine macrophage RAW 264.7 cells. MMP-9 expression and its biologic activity were blocked by inhibitors of TLR-9, ERK, p38 MAPK, and NF-κB, indicating that TLR-9 dependent ERK and p38 MAPK activation and the subsequent NF-κB activation are required for the CpG ODN-induced MMP-9 expression.

Materials and Methods

Reagents and antibodies

Oligodeoxynucleotide (ODN) 1688, 5'-TCCATGACGTTCCTGATGCT-3' and ODN1722, 5'-TCCATGAGCTTCCTGATGCT-3' were synthesized by Xenotech (Daejoun, Korea), and endotoxin content of these oligonucleotides was measured using a chromogenic limulus amoebocyte lysate kit (BioWhittaker, Walkersville, MD). MAPK inhibitors, U0126 and SB203580, and NF-κB inhibitor, SN-50, and endosomal maturation inhibitor, bafilomycin A, were obtained from Calbiochem (San Diego, CA) and chloroquine was purchased from Sigma-Aldrich Co. (St. Louis, MO). For Western blot analysis, we used Abs against ERK1/2, p38 MAPK, NF-κB and IκBα (Cell Signaling Technology, Beverly, MA), and MMP-9 (Santa-Cruz Biotechnology, Santa-Cruz, CA). Peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, or anti-mouse IgG (Santa-Cruz Biotechnology, Santa-Cruz, CA) were used as secondary antibodies.

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection and were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD) containing 10% FBS, 2 mM L-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37°C in 5% CO_2 in a water-saturated atmosphere.

Gelatin zymography

MMP-9 activity was evaluated using gelatin zymography employing 8% SDS-polyacrylamide gel containing 0.1% gelatin as a substrate. After electrophoresis, gels were washed three times with 2.5% Triton X-100 solution and then incubated overnight at 37°C in 0.2% Brij 35, 5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris, at pH 7.4, in a closed container. Gels were then stained for 30 min with 0.25% Coomassie R-250 in 10% acetic acid and 45% methanol and then destained for 30 min using an aqueous mix of 20% acetic acid, 20% methanol, and 17% ethanol. Areas of MMP-9 activity appeared as clear bands.

Western blot analysis

RAW 264.7 cells were stimulated with 1 μM ODN 1688, washed twice in cold PBS, and lysed on ice using lysis solution (1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail). Protein concentrations were determined using Bio-Rad protein assays. Briefly, proteins from cell lysates (50 μg) were boiled at 95°C in Laemmli SDS loading buffer, separated on 8% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in Tris buffered saline-0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary antibody for 4 h at room temperature. After 5 × 10 min washes in TTBS, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h. Following 5 additional 10 min washes with TTBS, protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham).

Establishment of stable cell line containing NF-κB luciferase reporter construct

A promoter-reporter construct containing 8 copies of the NF-κB element was transfected into RAW 264.7 cells using Lipofectamine 2000. After transfection, cells were incubated in complete media for 24 h at 37°C, and transfectants were selected under 400 μg/ml G418. Established RAW/NF-κB cells were stimulated with CpG ODN for 6 h at 37°C. In some experiments, cells were preincubated with a specific

inhibitor for 1 h at 37°C prior to CpG ODN stimulation. Cell lysates were assayed for luciferase activity using a luminometer (Promega) according to the manufacturer's instructions.

Results

CpG ODN-mediated activation of ERK1/2 and p38 MAPK is required for the MMP-9 induction

Previously, we reported CpG ODN-induced MMP-9 expression. RAW 264.7 murine macrophage cells were stimulated with ODN1688 or ODN1720, which has the same sequence as ODN1688 with a GpC dinucleotide replacing the CpG motif as a negative control. Conditioned media were collected and examined for a MMP-9 expression using gelatin zymography and western blot analysis. While ODN1720 failed to induce MMP-9, dose and time-dependent MMP-9 expression was observed and detected as early as 6 h post CpG ODN stimulation and reached a maximum level up to 24 h later. We further observed that CpG ODN induces transcriptional up-regulation and *de novo* protein synthesis of MMP-9 (Lim *et al.,* 2006).

A number of studies showed that activation of MAPK is essential for MMP-9 expression in various cell types. We examined CpG ODN-induced activation of MAPK and its involvement in MMP-9 expression. We tested the effect of MAPK inhibitors on CpG ODN-mediated MMP-9 induction. Prior to CpG ODN treatment, RAW 264.7 cells were pretreated with U0126 and SB203580, which are ERK, p38 MAPK inhibitors, respectively. CpG ODN-induced MMP-9 release into culture media was determined by gelatin zymograph and Western blot analysis. As shown in Figure 1A, inhibition of either ERK or p38 MAPK resulted in a significant decrease in MMP-9 expression from CpG ODN-stimulated cells. While SP600125, JNK inhibitor, had no effect on CpG ODN-mediated MMP-9 expression (data not shown), indicating that activation of ERK and p38 MAPK, but not JNK, is required for the CpG ODN-induced MMP-9 expression.

Since our previous data showed that MMP-9 expression by CpG ODN precedes TNF-α induction, we next examined whether inhibition of ERK or p38 MAPK has an effect on the CpG ODN-induced TNF-α production which leads to MMP-9 expression. As seen in Figure 1B, TNF-α production in response to CpG ODN was significantly reduced in the pre-

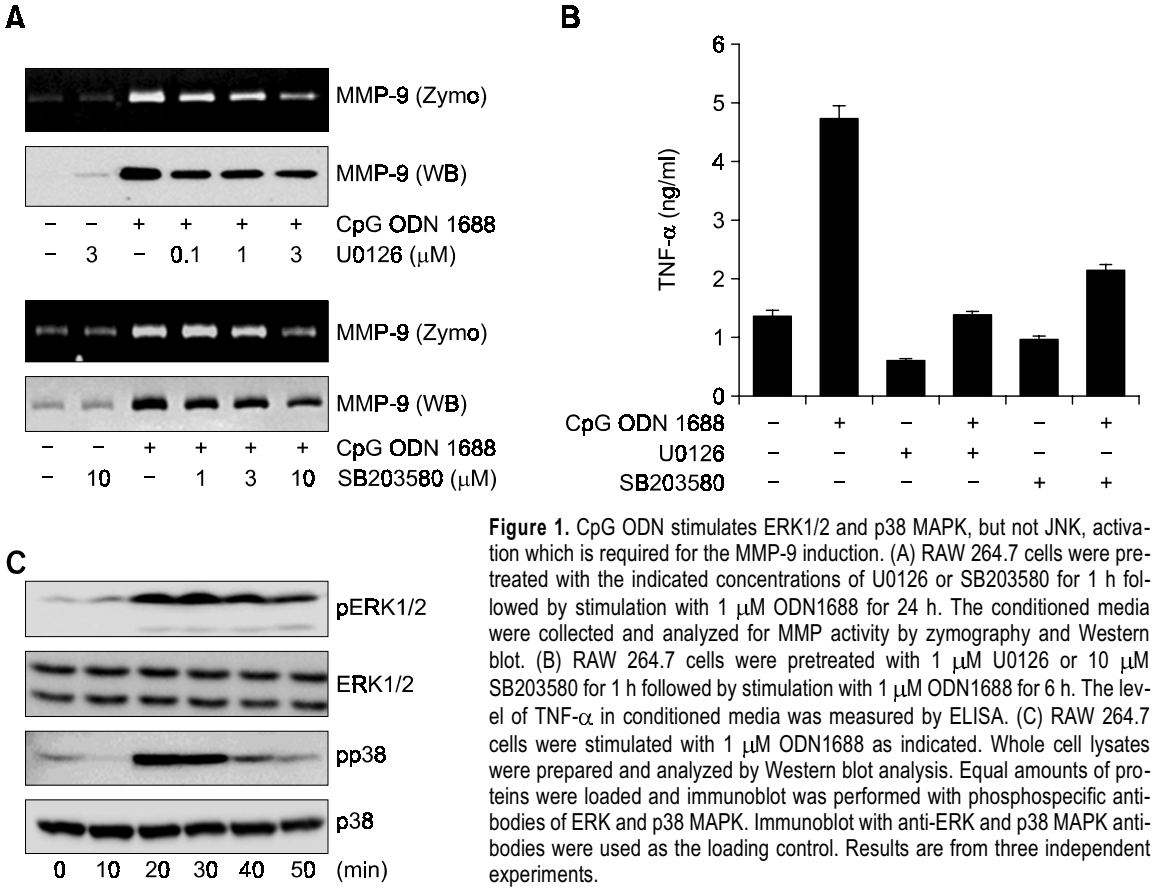

Figure 1. CpG ODN stimulates ERK1/2 and p38 MAPK, but not JNK, activation which is required for the MMP-9 induction. (A) RAW 264.7 cells were pretreated with the indicated concentrations of U0126 or SB203580 for 1 h followed by stimulation with 1 μM ODN1688 for 24 h. The conditioned media were collected and analyzed for MMP activity by zymography and Western blot. (B) RAW 264.7 cells were pretreated with 1 μM U0126 or 10 μM SB203580 for 1 h followed by stimulation with 1 μM ODN1688 for 6 h. The level of TNF-α in conditioned media was measured by ELISA. (C) RAW 264.7 cells were stimulated with 1 μM ODN1688 as indicated. Whole cell lysates were prepared and analyzed by Western blot analysis. Equal amounts of proteins were loaded and immunoblot was performed with phosphospecific antibodies of ERK and p38 MAPK. Immunoblot with anti-ERK and p38 MAPK antibodies were used as the loading control. Results are from three independent experiments.

sence of either U0126 or SB203580, suggesting that both ERK and p38 MAPK activation are required for the CpG ODN-mediated TNF-α production, which influences the subsequent MMP-9 expression.

To confirm the involvement of ERK and p38 MAPK activation in CpG ODN induced MMP-9 expression, phosphorylation of these kinases by CpG ODN was observed. ERK phosphorylation was detected at 20 min after CpG ODN stimulation and sustained up to 50 min. It seems that p38 MAPK phosphorylation is a bit temporal, since its phosphrylation was found at 20-30 min and declined to almost basal level by 40 min (Figure 1C).

CpG ODN-mediated activation of ERK and p38 MAPK is TLR-9 dependent

Internalization and endosomal maturation have been shown to be required for CpG DNA to activate TLR9 signaling in immune cells (Krieg, 1999; Ahmad-Nejad *et al.*, 2002). We examined if TLR-9 is responsible for the CpG ODN mediated ERK and p38 MAPK activation. Both chloroquine and bafilomycin A which effectively block endosomal maturation, significantly inhibited the phosphorylation of ERK and p38 MAPK by CpG ODN (Figure 2A), which resulted in the suppression of MMP-9 expression (Figure 2B). These results imply that CpG ODN elicits TLR-9 dependent activation of ERK and p38 MAPK and the subsequent MMP-9 expression.

NF-κB activation is involved in CpG ODN-mediated MMP-9 induction

Since NF-κB is one of the well known transcription factors which regulate MMP-9 expression, we addressed involvement of NF-κB activation in CpG ODN-mediated MMP-9 induction. RAW 264.7 cells were treated with 1 μM ODN1688 for 10 to 60 min and whole cell lysates were prepared from those cells. IκBα degradation was determined by Western blot analysis using a specific antibody against the IκBα subunit. The level of IκBα was decreased at 20-30 min after stimulation and then recovered to baseline status 40 min later (Figure 3A).

Next, we transfected NF-κB-luciferase reporter

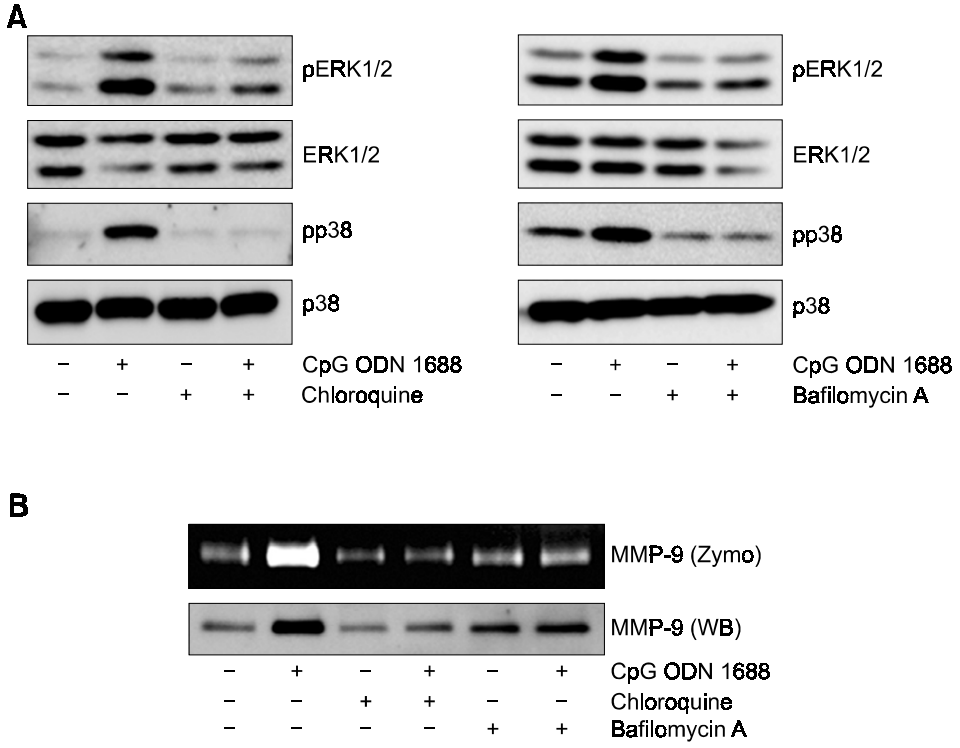

Figure 2. TLR9 inhibitors block CpG ODN induced ERK and p38 MAPK activation and MMP-9 expression. (A) RAW 264.7 cells were pretreated with 3 μM chloroquine or 10 μM bafilomycin A for 1 h followed by stimulation with 1 μM ODN1688 for 20 min. Cells were lysed and analyzed by Western blot analysis using phosphospecific antibodies of ERK and p38 MAPK. (B) RAW 264.7 cells were pretreated with 3 μM chloroquine or 10 μM bafilomycin A for 1 h followed by stimulation with 1 μM ODN1688 for 24 h. The conditioned media were collected and analyzed for MMP activity by zymography and Western blot using MMP-9 antibody. Results are from three independent experiments.

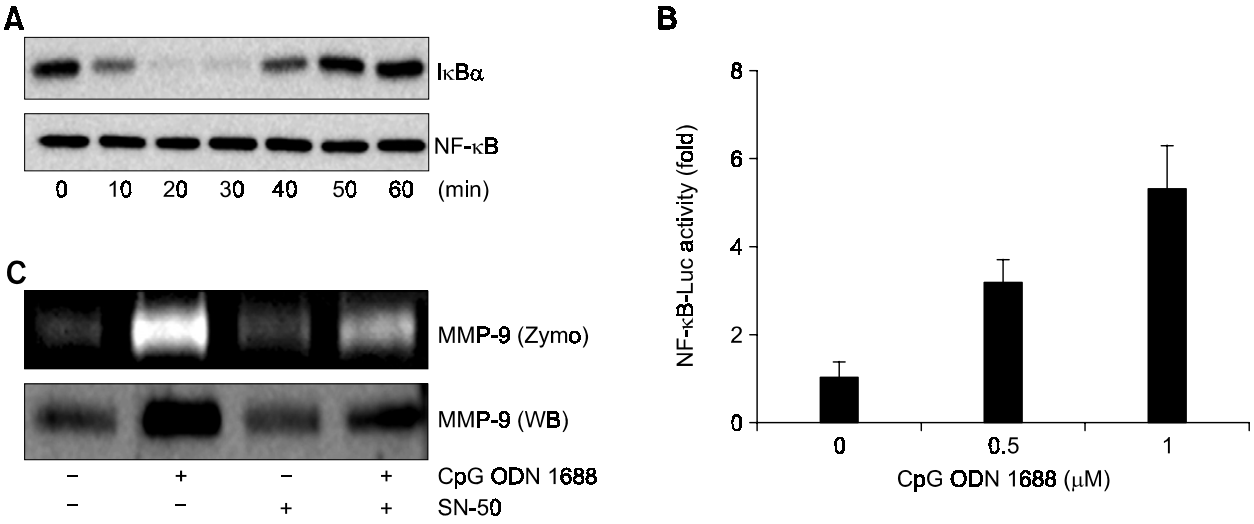

Figure 3. CpG ODN induces NF-κB activation and NF-κB inhibitor blocks MMP-9 induction. (A) RAW 264.7 cells were stimulated with 1 μM ODN1688 as indicated. Cells were lysed and analyzed by Western blot analysis using IκBα antibody. (B) RAW 264.7 cells expressing NF-κB luciferase reporter construct were treated with the indicated concentrations of ODN1688 for 6 h. Whole cell lysate was prepared and subjected to luciferase assay. (C) Cells were pretreated with the indicated concentrations of IκB kinase inhibitor peptide followed by stimulation with 1 μM ODN1688 for 24 h. The conditioned media were collected and analyzed for MMP activity by zymography and Western blot. The data shown are representative of three independent experiments.

construct, which contains 8 consecutive NF-κB binding sites in the upstream of the luciferase gene, into RAW 264.7 cells to establish RAW 264.7/NF-κB. This allowed us to measure NF-κB activation as a function of luciferase activity. RAW 264.7/NF-κB cells were treated with different concentrations of ODN1688 (0, 0.5, 1 μM) for 6 h and then luciferase activity in whole cell lysates were measured. As shown in Figure 3B, 3.7 to 5.5 fold-increased luciferase activities were observed after CpG ODN treatment, suggesting NF-κB activation by CpG ODN.

To further confirm CpG ODN-mediated NF-κB activation, the effect of SN-50, NF-κB inhibitor, on MMP-9 induction was examined. RAW 264.7 cells were stimulated with 1 μM ODN1688 for 24 h in the absence or presence of 10 μg/ml SN-50. Gelatin zymography and Western blot analysis were performed with conditioned media. We found SN-50 blocked MMP-9 expression significantly, indicating that NF-κB activation is required for the CpG ODN-induced MMP-9 expression (Figure 3C).

NF-κB is the downstream target of p38 MAPK, not ERK1/2, in the CpG ODN-stimulated signaling pathway

Since both ERK and p38 MAPK activation and NF-κB activity are required for the CpG ODN-induced MMP-9 expression, we addressed the possible link between MAPK activation and NF-κB activity. As illustrated in Figure 4A, pretreatment of SB203580 prior to CpG ODN stimulation abrogated IκBα degradation, while U0126 failed to block ODN-induced IκBα degradation. We also found that NF-κB dependent luciferase activity in RAW 264.7/ NF-κB cells after ODN stimulation was suppressed in the presence of SB203580, but not U0126 (Figure 4B). These results indicate that NF-κB is the downstream target of p38 MAPK in the CpG ODN- mediated signal transduction, but not involved in ERK1/2 pathway.

Discussion

In the present study, we extended our previous observations of CpG ODN-induced MMP-9 expression in murine macrophage RAW 264.7 cells. We further found that the CpG ODN elicits TLR-9 dependent ERK and p38 MAPK activation and the subsequent NF-κB activation to produce MMP-9.

Investigators have reported that ERK1/2, JNK, and p38 MAPK are all involved in the induction of MMPs (Werle *et al*., 2002; Spallarossa *et al*., 2005). For example, Werles *et al*. (2002) showed that monocyte chemoattractant protein (MCP)-1 can lead to direct activation of MAPK together with induction of MMP2 in vascular endothelial cells. Our work showed that inhibition of ERK and p38 MAPK using U0126 and SB203580, respectively, prevents CpG ODN-induced MMP-9 expression. However, JNK inhibitor SP600125 failed to inhibit MMP-9 release after CpG ODN treatment, suggesting that activation of ERK and p38

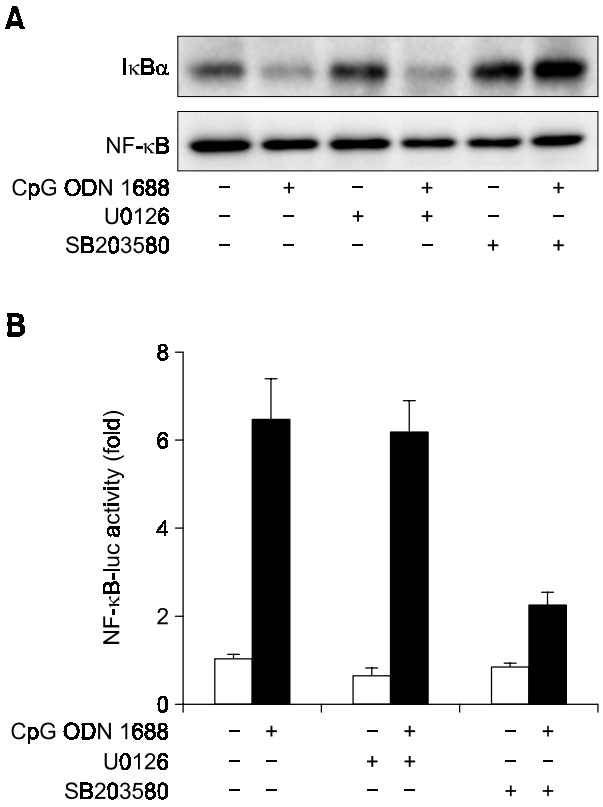

Figure 4. Inhibition of p38 MAPK, but not ERK1/2, prevents CpG ODN-induced NF-κB activation. (A) RAW 264.7 cells were pretreated with the indicated concentrations of 3 μM U0126, or 10 μM SB203580 for 1 h followed by stimulation with 1 μM ODN1688 for 20 min. Cells were lysed and analyzed by Western blot analysis using IκBα antibody. (B) RAW 264.7 cells expressing NF-κB luciferase reporter construct were treated with the 3 μM U0126 or 10 μM SB203580 for 1 h followed by ODN1688 for 6 h. Whole cell lysates were prepared and subjected to luciferase assay. The data shown are representative of three independent experiments.

MAPK, but not JNK, is critical for mediating CpG-induced production of MMP-9 from macrophage cells.

CpG ODNs have been shown to activate MAPK. Hacker *et al.* (1998) also reported that CpG ODN activates the MAPK kinase-ERK pathway in RAW 264.7 macrophage cell line, which plays an important role in IL-12 production from these cells. In contrast to RAW 264.7, they reported that CpG ODN activates JNK and p38 MAPK in bone marrow-derived dendritic cells. The role of JNK2 has been investigated in Th1 differentiation, and IL-12 fails to induce differentiation of JNK2-deficient $CD4^+$ T cells into effector Th1 cells (Dong *et al.*, 1998, 2000) and cytokine production from B cell lines and monocytic cell lines (Yi *et al.*, 1996, 1998). These observations suggest that ERK, JNK, and p38 MAPK-signaling pathways play an important role in the induction of immune responses by CpG ODN. In our present study, we also demonstrated that CpG phosphorylates ERK and p38 MAPK in RAW 264.7 cells.

The NF-κB responsive element is present in the promoter region of MMP-9. It has recently reported that CpG ODN regulates NF-κB-mediated-MMP-9 activation and controls cell migration (Rhee *et al.*, 2007). We also observed NF-κB activation by CpG ODN. As shown in Figure 3A and 3B, CpG ODN elicits IκBα degradation and increases luciferase activity in RAW 264.7 cells containing NF-κB-luciferase reporter construct. We further found that NF-κB inhibitor, SN-50, blocked MMP-9 expression, indicating that NF-κB activation is required for the CpG ODN-induced MMP-9 expression.

MAPK, either through direct or indirect interaction with transcription factors such as NF-κB, regulates various biological functions. To address whether MAPK activation is associated with NF-κB activity in CpG ODN-induced MMP-9 expression, RAW 264.7 cells were stimulated with CpG ODN in the absence or presence of U0126 and SB203580. We found that IκBα degradation and NF-κB dependent luciferase activity were suppressed by SB203580 pretreatment, indicating CpG ODN activates p38 MAPK/NF-κB/MMP-9 pathway in RAW 264.7 cells.

However, ERK activation by CpG ODN dose not seem to lead to NF-κB activation, since ERK inhibitor, U0126, has no effect on IκBα degradation and NF-κB dependent luciferase activity in response to CpG ODN. In addition to NF-κB binding site, MMP-9 promoter region contains AP-1 bindig site and a recent report showed that ERK/AP-1 signaling pathway is involved in the MMP-9 expression by hepatitis B virus X protein (Chung *et al.,* 2004). Therefore, we speculate that CpG ODN-induced ERK activation might lead to AP-1 activation which is followed by MMP-9 expression.

In summary, we demonstrate a mechanism of the CpG ODN-mediated MMP-9 expression in macrophage cells, which involves TLR9, ERK and p38 MAPK activation, and the subsequent NF-κB dependent transcriptional up-regulation.

Acknowledgement

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