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Activation of toll-like receptor-9 induces matrix metalloproteinase-9 expression through Akt and tumor necrosis factor-α signaling

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Abstract **CpG oligodeoxunucleotide (ODN) plays an important role in immune cell function. The present study examined whether temporal control of toll-like receptor (TLR)-9 by CpG ODN can regulate the expression of matrix metalloproteinase-9 (MMP-9). CpG ODN induced the release of tumor necrosis factor (TNF)-α and the expression of TNF receptor (TNFR)-II, but not of TNFR-I, in a time-dependent manner and stimulated significant, though delayed, MMP-9 expression. The endosomal acidification inhibitors, chloroquine or bafilomycin A, inhibited CpG ODN-induced TNF-α, TNFR-II, and MMP-9 expression. CpG ODN induced the phosphorylation of Akt, and the inhibition of Akt by LY294002 suppressed CpG ODN-induced TNF-α, TNFR-II, and MMP-9 expressions. Moreover, neutralizing TNF-α antibody significantly suppressed CpG ODN-induced MMP-9 expression, suggesting the involvement of TNF-α. These observations suggest that CpG ODN may play important roles in macrophage activation by regulating the expression of MMP-9 via a TLR-9/Akt/TNF-α-dependent signaling pathway.**

Keywords: CpG oligodeoxunucleotide; Matrix metalloproteinase; Toll-like receptor; Akt; Tumor necrosis factor

1. Introduction

Cells of the innate immune system respond to pathogens by recognizing conserved pathogen-associated molecular patterns [1]. Pathogen-associated molecular patterns include lipopolysaccharide and nucleic acids, such as viral DNA and bacterial DNA. Bacterial DNA induces interferon-γ production and activates NK cells, and B-cell and macrophages are subsequently identified as direct cellular targets of bacterial DNA [2–4]. The immunostimulatory activity of bacterial DNA requires an unmethylated CG dinucleotide within a certain base context (CpG motif). CpG oligodeoxynucleotide (ODN), a synthetic analog of bacterial DNA, is capable of activating innate immunity, including macrophages, and the recognition of CpG requires the expression of intracellular toll-like receptor (TLR)-9, which is constitutively expressed by macrophages [4–6]. Upon internalization, CpG binds to TLR-9 in early endosomes and triggers a signaling cascade mediated via MyD88 and NF-κB. Consequently, CpG-stimulated macrophages secrete tumor necrosis factor (TNF)-α and various cytokines as well as other factors involved in immune reactions [7,8].

TNF-α is the predominant cytokine during early response to acute extracellular infection and is an important mediator of inflammatory immune responses. There are two major cell surface receptors for TNF-α, type I TNF receptor (TNFR) and type II TNFR, both of which belong to the TNFR superfamily and bind TNF-α with high affinity [9,10]. Although binding of activated TNF-α to its receptors can result in the activation of signaling events, signaling triggered by TNFR-I and TNFR-II usually cause the activation of transcription factors, which then induce numerous genes, such as matrix metalloproteinase (MMP)-9, that are involved in inflammatory responses [11,12]. TNF-α-induced MMP-9 is associated with invasion. TNF-α is one of the key mediators of MMP-9 induction, because modulation of autocrine TNF-α directly stimulates MMP-9 expression [13,14], or TNF-α neutralizing antibodies or the inhibition of TNF-α-induced MMP activity by MMP inhibitors are effective in the treatment of rheumatoid arthritis [15]. However, although the roles of MMP in inflammatory cell functions have been examined, the predominant focus of attention has been the effect of MMPs on adhesion and migration. In addition, although many studies have emphasized the role of CpG ODN as a strong immunomodulator, no information is available regarding to its potential contribution to MMP-9 expression.

In this study, we investigated whether CpG ODN induces the phosphorylation of Akt in murine macrophage Raw264.7 cells, and whether Akt signaling events mediate the expressions of TNF-α, TNFR-II, and MMP-9.

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Abbreviations: CpG ODN, CpG oligodeoxynucleotide; TLR, toll-like receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3 kinase; MAPK, mitogen-activated protein kinase; ECM, extracellular matrix

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2. Materials and methods

2.1. Reagents and antibodies

Cell culture reagents, including FBS, were obtained from Life Technologies (Grand Island, NY), CpG ODN, gelatin, and chloroquine were from Sigma–Aldrich Co. (St. Louis, MO), TNF-α ELISA kits were from R&D Systems (Minneapolis, MN), polyclonal MMP-9 antibody from Cayman Chemical (Ann Arbor, MI), TNFR-I and -II antibodies from Santa-Cruz Biotechnology (Santa-Cruz, CA), and Akt and phospho-Akt antibodies from Cell Signaling (Beverly, MA). LY294002 and bafilomycin A were purchased from Calbiochem (San Diego, CA).

2.2. Cell culture

Murine macrophages, Raw264.7 cells (ATCC, CCL-2278), were grown in RPMI 1640 supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, and antibiotics. Culture dishes were maintained in a humidified 5% CO_2 at 37 °C.

2.3. Real-time PCR

Total RNA was extracted from cells using TRIzol reagent. One microgram of total RNA was used as a template to make first strand cDNA by random priming using the Promega reverse transcriptase system. Real-time PCR was performed using an iCycler real-time detection system using SYBR-Green I as the fluorescent dye, which enabled the real-time detection of PCR products according to the manufacturer's instructions. The synthetic gene-specific primer sets used for PCR were: (i) MMP-9 forward primer, 5′-CCT ACT CTG CCT GCA CCA CTA AA-3′, and reverse primer, 5′-CTG CTT GCC CAG GAA GAC GAA-3′, which amplified a 179-bp of mouse MMP-9 cDNA; (ii) TNF-α forward primer, 5′-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3′, and reverse primer, 5′-GTA TGA GAT AGC AAATCG GCT GAC GGT GTG GG-3′, which amplified a 354-bp of mouse TNF-α cDNA; (iii) TNFR-I forward primer, 5′-CCG GGC CAC CTG GTC CG-3′, and reverse primer, 5′-CAA GTA GGT TCC TTT GTG-3′, which amplified a 306-bp of mouse TNFR-I cDNA; (iv) TNFR-II forward primer, 5′-GTC GCG CTG GTC TTC GAA CTG-3′, and reverse primer, 5′-GGT ATA CAT GCT TGC CTC ACA GTC-3′, which amplified a 233-bp of mouse TNFR-II cDNA; (v) β-actin forward primer, 5′-TCC TTC GTT GCC GGT CCA CA-3′, and reverse primer, 5′-CGT CTC CGG AGT CCA TCA CA-3′, which amplified a 509-bp mouse β-actin cDNA. The PCR conditions were 94 °C for 3 min, followed by 40 amplification cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s). For quantification purposes, target genes were normalized versus β-actin.

2.4. Gelatin zymography

MMP activity was determined by gelatinase zymography using 0.1% gelatin as a substrate in 8% SDS–polyacrylamide gel. After electrophoresis, gels were washed three times with 2.5% Triton X-100 in water and then incubated overnight at 37 °C in 0.2% Brij 35, 5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris, 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 destained for 30 min using an aqueous mixture of 20% acetic acid, 20% methanol, and 17% ethanol. Areas of protease activity appeared as clear bands.

2.5. Western blot analysis

Cells were plated in wells (1×10^6) and treated with CpG ODN in the presence or absence of inhibitor. They were then washed with cold-phosphate buffered saline (PBS), trypsinized and pelleted at $700 \times g$. Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail). The preparations were then cleared by centrifugation and supernatants were saved as cell lysates. Proteins were separated by 8% reducing SDS–PAGE and immunoblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto nitrocellulose membranes. Membranes were then blocked with 5% non-fat dry milk in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween 20) and incubated with primary antibody for 4 h. Subsequently, membranes were washed, incubated for 1 h with secondary antibody conjugated to HRP, rewashed, and finally developed using an enhanced ECL system.

2.6. Akt phosphorylation assays

Cells were treated with CpG ODN in the presence or absence of various inhibitors, lysed with lysis solution, and Western blotted as described above. The membranes so obtained were probed with antibody against active (phosphorylated) Akt, and blots were stripped and reproved with antibody recognizing both active and total Akt, to demonstrate equal loading.

2.7. Detection of soluble TNF-α

Raw264.7 cells were incubated for 6 h in CpG ODN alone or in CpG ODN containing various inhibitors. At designated time, conditioned media were harvested and assayed for TNF-α in triplicate wells by ELISA according to the manufacturer's protocol. The data shown represent at least three independent experiments.

3. Results

3.1. CpG ODN enhanced the expressions of TNF-α and TNFR-II, but not of TNFR-I

CpG ODN enhanced proinflammatory cytokine TNF-α secretion by Raw264.7 macrophages. Because cellular activation increased TNFR expression, and high levels of TNF-α secretion increased the expression of its receptor [12,16], we postulated that CpG ODN would stimulate TNFR expression. To confirm that the above results were reproduced in Raw264.7 cells, similar experiments were performed. Cells were treated with CpG ODN for various times, and TNF-α secretion and TNFRs expressions subsequently measured. Like TNF-α, TNFR-II expression was increased in cell lysates after Raw264.7 cells had been stimulated with CpG ODN (Fig. 1A and B). In contrast, the expression of TNFR-I did not change in response to CpG ODN (data not shown). In addition, the mRNAs of TNF-α, TNFR-II and MMP-9 were also increased by CpG ODN stimulation, although their kinetics differed (Fig. 1C). Especially, CpG ODN induced the expression of TNF-α mRNA earlier than MMP-9 mRNA.

3.2. TLR-9 was involved in CpG ODN-induced response

RT-PCR confirmed the presence of TLR-9 in Raw264.7 cells (data not shown), which is agreement with the previous report [17]. The antimalarial drug chloroquine has been shown to inhibit proinflammatory responses during endosomal maturation [18], and recent evidence suggests that chloroquine also inhibits TLR-9-mediated innate immune activation [19]. To examine the effect of chloroquine on CpG ODN-induced gene expression, macrophages were stimulated with CpG ODN in the presence of chloroquine. CpG ODN-induced TNF-α, TNFR-II, and MMP-9 expressions were diminished by chloroquine (Fig. 2A). In addition, the pretreatment of macrophages with the other endosomal acidification inhibitor, bafilomycin A [20] also completely inhibited CpG ODN-induced MMP-9 expression and the expression of TNF-α and TNFR-II (Fig. 2B), suggesting that chloroquine or bafilomycin A may inhibit CpG ODN-induced TLR-9-mediated innate immune response.

3.3. CpG ODN-induced MMP-9 expression via the Akt pathway

Recently, a study suggested that CpG ODN induces the phosphorylation of Akt [21]. However, the molecular mechanism underlying the involvement of Akt in MMP-9 expression has not been studied. Since Akt has been implicated in the induction of MMP-9, we examined the effect of CpG ODN

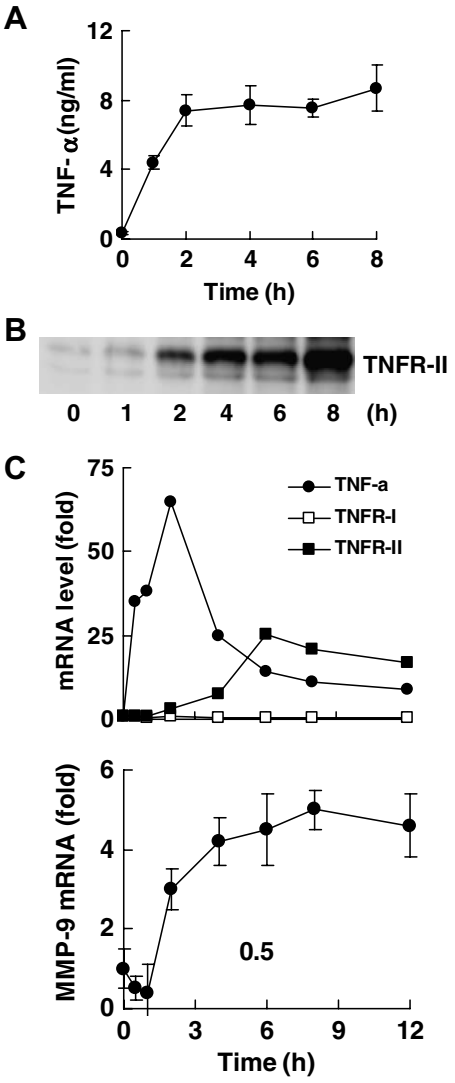

Fig. 1. CpG ODN-induced TNF-α, TNFR-II and MMP-9 expression but not TNFR-I. (A and B) Following overnight serum starvation, cells were treated with 1 μM of CpG ODN for the indicated times. Conditioned media were collected at various times and TNF-α amounts were determined by ELISA. Whole cell lysates were used for Western blot analysis of TNFR-II. (C) Cells were cultured with CpG ODN (1 μM) for the indicated times and total RNA was isolated from cells and then subjected to real-time PCR. The graph represents fold changes of TNF-α, TNFR-I, TNFR-II, and MMP-9 mRNA after cells were treated with CpG ODN.

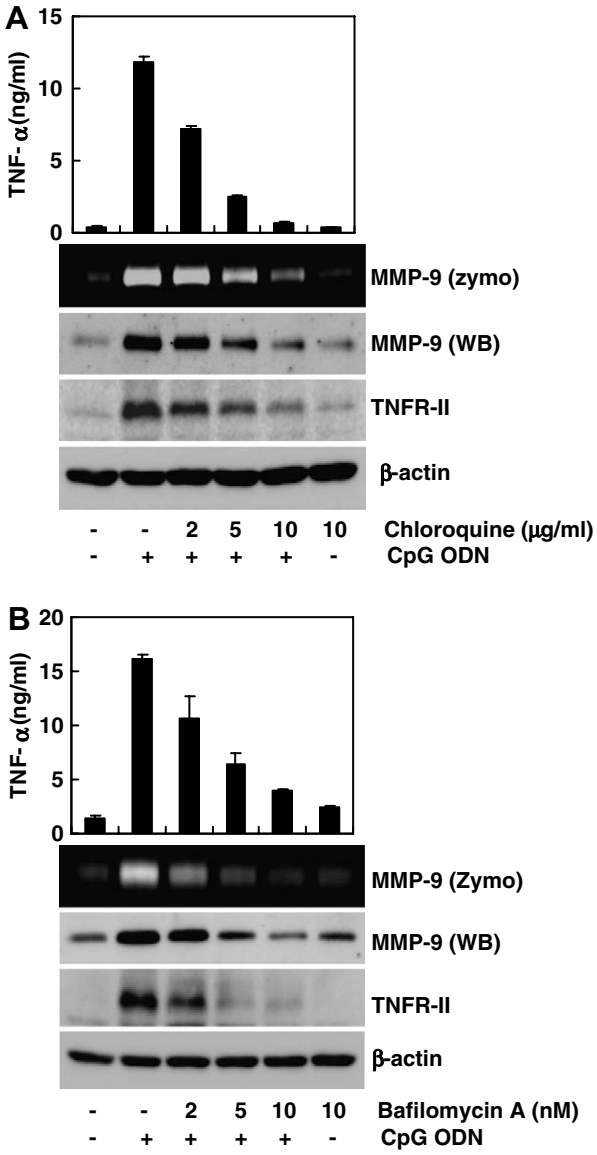

Fig. 2. TLR-9 activation mediated CpG ODN-induced MMP-9 expression. Macrophages were treated with CpG ODN (1 μM) in the absence or presence of the indicated doses of chloroquine (A), or bafilomycin A (B). Conditioned media were collected at 6 h and analyzed for TNF-α, and portions were concentrated and analyzed for MMP-9 expression by Zymography and Western blotting. Whole cell lysates were used for the Western blot analysis of TNFR-II, as indicated. Results shown are representative of two separate experiments.

on Akt activation, by using Western blot analysis with anti-phospho-specific antibody against Akt. When macrophages were stimulated with 1 μM CpG ODN for different times, the phosphorylation level of Akt was transiently increased, peaking after 30 min of stimulation and then decreasing at 50 min (Fig. 3A). To determine whether the activation of Akt plays a role in the regulation of MMP-9, cells were pre-treated with a selective inhibitor of the phosphatidylinositol 3 kinase (PI3K)-Akt pathway for 30 min and then treated with CpG ODN for 30 min or 6 h. As shown in Fig. 3B and C, the inhibition of Akt phosphorylation with LY294002 reduced CpG ODN-induced MMP-9 expression, and in agreement with this result, we found that LY294002 inhibited MMP-9 gelatinolytic activity. To confirm whether the activation of Akt plays a role in the regulation of MMP-9 expression, we measured the mRNA level of MMP-9 in the presence of PI3K/Akt inhibitor, and found that LY294002 strongly inhibits its MMP-9 mRNA (Fig. 3D). In addition, the inhibition of PI3K/Akt with LY294002 reduced CpG ODN-induced TNF-α production and TNFR-II expression (Fig. 3E). As a control, we tested LY294002 effect in response to LPS because previous report suggested that PI3K-Akt pathway negatively regulates TNF-α production in response to LPS in monocytic cells [22]. The inhibition of PI3K/Akt pathway by LY294002 did not decrease LPS-induced TNF-α production (Fig. 3F), suggesting the role of PI3K/Akt pathway different from CpG ODN or LPS responses.

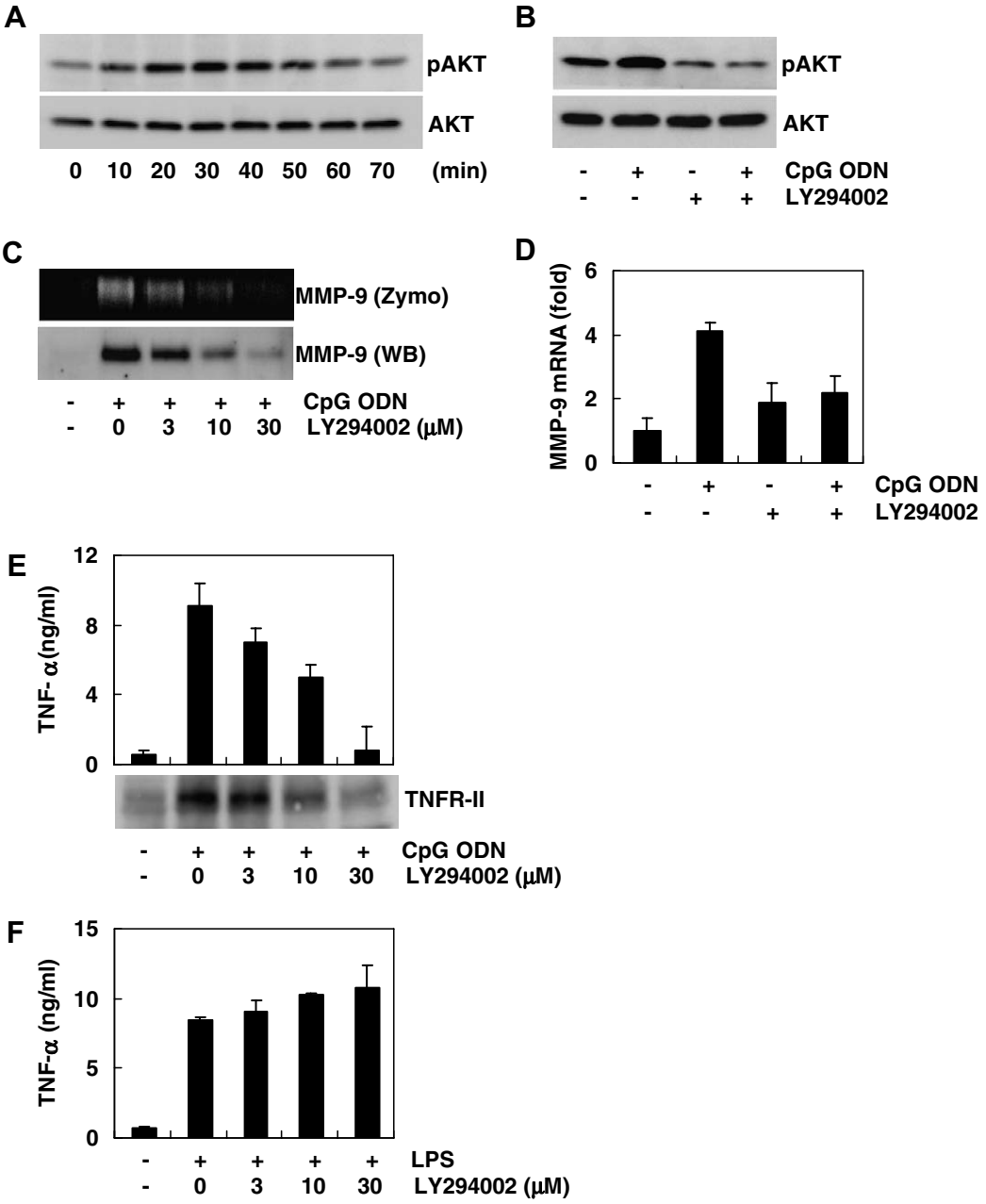

Fig. 3. Akt is a mediator of CpG ODN response. (A) Raw264.7 cells were treated with 1 μM CpG ODN for the indicated times. (B) Cells were pretreated with 1 μM CpG ODN in the absence or presence of LY294002. To detect Akt phosphorylation, equal amounts of cell extracts were analyzed by Western blotting using phospho-specific Akt antibody. As a loading control, the same blots were reprobed with anti-Akt antibody. (C) Cells were pretreated with the indicated doses of LY294002 for 1 h and stimulated with 1 μM CpG ODN. Conditioned media were collected at 6 h and analyzed for MMP-9 expression and activity by zymography and Western blotting. (D) Cells were pretreated with 10 μM of LY294002 for 1 h and then stimulated with 1 μM CpG ODN. Total RNA was isolated and quantitative real-time PCR was used to determine MMP-9 mRNA levels. (E and F) Cells were pretreated with the indicated doses of LY294002 for 1 h and stimulated with 1 μM CpG ODN or 1 μg/ml LPS. Conditioned media were collected and TNF-α levels were determined by ELISA. Whole cell lysates were used for the Western blot analysis of TNFR-II, as indicated.

3.4. TLR-9 and Akt are involved in TNF-α production before MMP-9 expression

Since our data show that CpG ODN activates Akt phosphorylation, we investigated the possibility of a relationship between TLR-9 and Akt, and we found that TLR-9 inhibition affects Akt phosphorylation. Macrophages were stimulated with CpG ODN in the absence or presence of chloroquine, and Western blot analysis showed that CpG ODN-induced Akt phosphorylation was blocked by pretreating chloroquine, TLR-9 inhibitor. Moreover, the phosphorylation of Akt in cells by CpG ODN was also significantly suppressed in the presence of bafilomycin A (Fig. 4A), indicating that Akt is a downstream kinase of TLR-9. Furthermore, our data demonstrate that CpG ODN induces the expression of TNF-α mRNA earlier than MMP-9 mRNA. Experiments were therefore performed to determine if CpG ODN exerts its effects through the production of TNF-α. To examine whether CpG ODN-induced MMP-9 expression by macrophages is TNF-

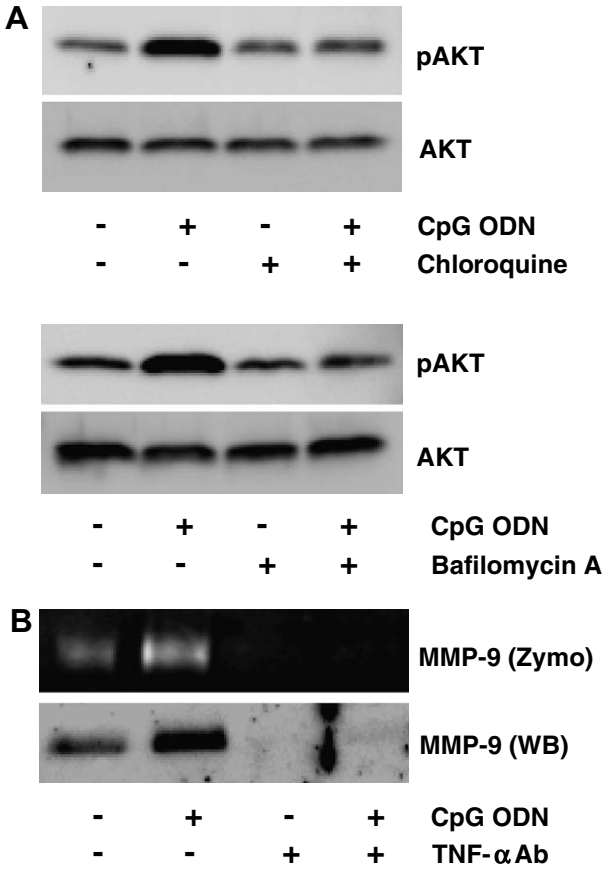

Fig. 4. CpG ODN-induced MMP-9 expression through the activation of the TLR-9/Akt/TNF-α pathway. (A) Cells were pretreated with chloroquine (10 μg/ml) or bafilomycin A (10 nM) for 30 min before CpG ODN stimulation for 30 min. To detect Akt phosphorylation, equal amounts of cell extracts were analyzed by Western blotting using phospho-specific Akt antibody. As a loading control, the same blots were reprobed with anti-Akt antibody. (B) Cells were pretreated with neutralizing anti-TNF-α antibody (1 μg/ml) for 30 min before being treated with CpG ODN (1 μM) for 6 h. Following incubation, conditioned media were collected and analyzed for MMP expression and activity by Western blotting and zymography.

α-dependent, cells were pretreated with anti-TNF-α neutralizing antibody. Consistent with previous results, MMP-9 was found to be increased in the conditioned media of cells after stimulation with CpG ODN. However, cells treated with anti-TNF-α antibody failed to produce MMP-9 in response to CpG ODN (Fig. 4B), which indicates that CpG ODN-induced MMP-9 expression by macrophages requires TNF-α production.

4. Discussion

Previous reports have demonstrated that bacterial DNAs are immunostimulatory and that specific nucleotide sequences containing unmethylated CpG ODN are responsible for this activity [2–5]. TLR-9 has been identified as a receptor of CpG ODN. Many researches for CpG ODN have focused upon the roles of adaptor proteins and downstream kinases that lead to the activations of transcription factors. Although many proteins directly or indirectly participate in innate immune responses, little effort has been directed at understanding the mechanism of CpG ODN-mediated MMP-9 expression. We propose a mechanistic model of CpG ODN-induced TLR-9 binding and Akt activation, which result in TNF-α-mediated MMP-9 expression.

Both mitogen-activated protein kinase (MAPK) and Akt can stimulate MMP-9 expression. Previous studies have suggested a requirement for an intracellular MAPK-dependent step for CpG ODN-mediated cellular activation [23]. Moreover, studies on macrophages have demonstrated the CpG ODN-induced activation of various kinases [24]. Akt activation could also explain, at least in part, the role of TLR-9 in CpG ODN-mediated MMP-9 expression. It has been reported that CpG ODN-driven cell survival through the activation of the PI3K-Akt pathway plays a role in regulating immune response [25,26]. Our findings show that the expression of MMP-9 is strongly impaired by inhibitor of the PI3K-Akt pathway. Therefore, these results suggest that TLR-9 activation by CpG ODN regulates MMP-9 expression via a PI3K-Akt-dependent pathway.

Previous studies have suggested a requirement for an intracellular pH-dependent step in the CpG ODN-mediated activation of macrophages [20]. Especially, endosomal acidification is thought to be a critical step in the initiation of intracellular CpG ODN signaling through the binding of TLR-9 [18–20,27]. Our results indicate that the expression of MMP-9 by CpG ODN also involves this pathway. Pretreatment with bafilomycin A (an inhibitor of vacuolar H^+-ATPase) or chloroquine (which accumulates in and increases the pH within endosomes) strongly blocked CpG ODN-induced MMP-9 expression. Moreover, since TLR-9 is known to be a receptor of CpG ODN, it is believed that TLR-9 is required for the activation of Akt by CpG ODN. Some study suggested a TLR-independent mechanism of CpG ODN-induced cell activation [20,28]. However, the above studies suggest that TLR-9 is an important receptor in the process leading to MMP-9 expression by CpG ODN.

Hallmarks of chronic inflammation include the accumulation of inflammatory cells and the increased degradation of extracellular matrix (ECM) components. During tissue remodeling, increased ECM turnover can result in the release of degraded ECM components. TNF-α has been shown to be elevated in many chronic inflammatory diseases. Although TNF-α has a central role in the innate immune system for protection against infections when produced in excess, TNF-α may also orchestrate a chronic inflammatory response that leads to severe tissue damage. Previously, it was reported that CpG ODN can induce TNF-α by macrophages [29,30]. Here, we found that CpG ODN induces the expression of MMP-9 by macrophages. Because TNF-α can induce MMP-9, we examined whether CpG ODN activates macrophage MMP-9 expression via TNF-α-dependent signaling. Our results show that CpG ODN increases the expression of TNF-α prior to MMP-9 production in macrophages, and that TNF-α signaling, specifically through TNFR-II, is required for MMP-9 expression. Macrophages express surface TNFRs and respond to TNF-α, and MMP-9 expression by macrophages does require new protein synthesis, suggesting that the CpG ODN-induced expression of MMP-9 by macrophages is dependent on TNF-α production. This hypothesis is supported by the finding that pretreatment with neutralizing TNF-α antibody strongly blocked basal and CpG ODN-induced MMP-9 expression.

In our system, basal TNF-α amounts and MMP-9 activity were showed in the unstimulated macrophages during cell culture. These observations suggest that basal TNF-α contributes to basal expression of MMP-9. In addition, isotype matched antibody with neutralizing TNF-α antibody had no effect on MMP-9 expression, excluding the possible effect of non-specificity of neutralizing antibody.

In this study, we examined the CpG ODN-induced mechanisms in macrophages that control MMP-9, which is involved in macrophage migration and immune response. The results presented in this study show that CpG ODN induces TNF-α and TNFR-II at the transcriptional level, and that these are involved in MMP-9 expression in supernatants derived from mouse cell line by a TLR-9 and Akt-mediated mechanism.

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