CpG DNA-mediated immune response in pulmonary endothelial cells

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Although the CpG DNA immune response mediated by Toll-like receptor 9 (TLR9) has been extensively studied in a number of immune cells, the response to CpG DNA in endothelial cells (EC) is not well understood. In this study, we show that both mouse and rat lung EC display constitutive expression of TLR9 mRNA. Exposure to CpG DNA induced a potent proinflammatory response as manifested by an increased expression of IL-8 and ICAM-1 in mouse pulmonary EC. The proinflammatory response was sensitive to chloroquine, consistent with a role of endosomal contribution. A role for p38 MAPK and NF-κB pathway was apparent as the response was sensitive to inhibitors of p38 MAPK and NF-κB but was not affected by inhibitors of ERK1/2. A synergistic effect of CpG DNA and LPS on the inflammatory response is consistent with multiple TLR interaction in EC. This study suggests a possible role for CpG DNA-mediated EC immune response in the host defense system. It also has important implications in plasmid DNA-mediated pulmonary endothelium gene transfer.

CpG motif; lung

THE VASCULAR ENDOTHELIUM SERVES as the key barrier between the intravascular compartment and extravascular tissues and plays a critical role in a large number of physiological and pathological processes (8, 19). As a critical part of inflammation, endothelial cells (EC) can recognize the molecular patterns commonly associated with many microbial agents and subsequently initiate the transcription of inflammatory genes (23, 29). Specific conserved components of microbes include lipopolysaccharides (LPS) from gram-negative bacteria, CpG DNA, and flagellin (11, 21, 22). A large body of evidence has shown that LPS is highly proinflammatory and elicits a wide array of immune responses in EC (1, 10). Little information is available on the direct biological effect of bacterial CpG DNA on the vascular EC.

Unlike LPS, which is recognized by Toll-like receptor (TLR) 4, CpG DNA is recognized by TLR9 (14). The signaling of these two receptors, however, shares similar downstream pathways, including activation of kinases like the stress kinases c-Jun NH2-terminal kinase, p38, and the IkB kinase (IKK) complex (25). A number of studies have shown that CpG DNA has strong stimulatory effects on murine and human lymphocytes in vitro and murine lymphocytes in vivo (5, 13, 18). These stimulatory effects include triggering B cell proliferation, resistance to apoptosis, and release of IL-6 and IL-12; natural killer cell secretion of IFN-γ and increased lytic activity; and monocyte/macrophage secretion of IFN-α/β, IL-6, IL-12, granulocyte-monocyte colony-stimulating factor, chemokines, and TNF-α. A recent study has shown that human colonic epithelial cells can also respond to CpG DNA via upregulation of IL-8, suggesting a new mechanism for the epithelial defense system against microbial agents (2). The purpose of the current study was to determine the CpG-mediated inflammatory responses in pulmonary EC.

In the present study, we provide evidence to demonstrate for the first time that mouse and rat EC constitutively express TLR9 mRNA. Exposure to CpG DNA induced a potent proinflammatory response as manifested by an increased expression of IL-8 and ICAM-1 in EC. We further show that LPS and CpG DNA have a synergistic effect in inducing inflammatory responses in EC. This study suggests a possible role for CpG DNA-mediated EC immune response in the host defense system. It also has important implications in plasmid DNA-mediated pulmonary endothelium gene transfer.

MATERIALS AND METHODS

Cell culture. Mouse lung endothelial cells (MLEC) were prepared as described previously (28) and were cultured in OPTI-MEMI supplemented with 20% (vol/vol) FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), and EC growth supplement (25 μg/ml) and 20% (vol/vol) FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), and 10 mM HEPES. Mouse macrophage cell line RAW 264.7 purchased from American Type Culture Collection (Manassas, VA) was cultured in DMEM supplemented with 10% (vol/vol) FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), and 10 mM HEPES. All cultures were incubated at 37°C in humidified atmosphere with 5% CO2.

Reagents. LPS was purchased from Sigma-Aldrich (St. Louis, MO). Two different types of CpG oligodeoxynucleotides (ODNs), 1668 (7) and 2006 (7), were used in this study, and their sequences are 5′-TCCATGACGTTTCTGAGTC-3′ and 5′-TCGTCGTTTGTCCCTGTGTT-3′, respectively. The sequence of non-CpG ODN used in this study is 5′-GCTTGATGACTCAAGCGGAAA-3′. All ODNs were synthesized by MWG (High Point, NC). SN-50, PD-98059, and SB-203580 were purchased from Calbiochem (San Diego, CA). Pyrrolidine dithiocarbamate (PDTC) and chloroquine were purchased from Sigma.

RT-PCR for TLR9, IL-8, and ICAM-1. Total RNA was extracted from cells with TRizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized with 1 μg of total RNA in a total reaction volume of 20 μl with SuperScript III Reverse Transcriptase (Invitrogen). cDNA MATERIALS AND METHODS

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whether CpG DNA can induce immune response in these cells. After demonstrating constitutive expression of TLR9 in mouse and rat lung EC, we then examined but not rat lung EC.

Expression of TLR9 mRNA in EC. RT-PCR analysis of RNA from MLEC, RPAEC, and RPMVEC displayed constitutive expression for TLR9, although their levels were lower than that in mouse RAW 264.7 macrophage cells. In contrast, rat pulmonary arterial smooth muscle cells showed no expression of TLR9 mRNA (Fig. 1).

Table 1. Sequences of the primers used for PCR

<table>
<thead>
<tr>
<th>Product</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Mouse TLR9</td>
<td>5’-GCT TGG GCC TTT CAC TGT TG-3’ (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-AAG TCG GCT CTG TGG AT-3’ (reverse)</td>
</tr>
<tr>
<td>Rat TLR9</td>
<td>5’-GCT TGG GCC TTT CAC TGT TG-3’ (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-AAG CCG GCT CTG CTT AT-3’ (reverse)</td>
</tr>
<tr>
<td>Mouse IL-8</td>
<td>5’-CTG CAG CCA CAC TCC AGA AGA-3’ (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-CAA CCT GAC GAA ACA AAG GG-3’ (reverse)</td>
</tr>
<tr>
<td>Mouse ICAM-1</td>
<td>5’-CTG CAG GAA CAA ACA ACA GG-3’ (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-CAA CCA AGA GTA CTT GCG CTC AGG-3’ (reverse)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-CCA AGA GTA CTT CTT GCT CTC AGG-3’ (reverse)</td>
</tr>
</tbody>
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TLR, Toll-like receptor.

(2 μl) was amplified with Taq DNA polymerase (Promega). For IL-8, ICAM-1, and β-actin, PCR reaction was performed for 30 cycles, each cycle consisting of denaturation (95°C for 50 s), annealing (56°C for 50 s), and an elongation step (72°C for 7 min). Amplification of mouse and rat TLR9 cDNA was performed by a similar protocol, except that annealing was set at 60°C. Primer sequences are given in Table 1. PCR products were separated on 2% agarose gels containing ethidium bromide and were visualized and photographed under ultraviolet (UV) light.

IL-8 (KC) ELISA. Cells were cultured in six-well cell culture plates until 80% confluent and then challenged with different treatments for indicated times. Medium was collected and kept frozen until use. The levels of mouse IL-8 (KC) were determined with a specific ELISA kit following the manufacturer’s instructions (R&D, Minneapolis, MN).

Immunocytochemistry. MLEC cultured in a 24-well plate were treated with CpG ODN (10 μg/ml) or non-CpG ODN (10 μg/ml) overnight. Controls included LPS (10 ng/ml) and PBS. Cells were then washed three times with ice-cold PBS and fixed in 0.25 ml of ice-cold 4% paraformaldehyde for 5–10 min. Cells were washed in PBS (3×, 4°C) and were incubated with blocking solution (1% goat serum in PBS) for 1 h at 4°C. Cells were then incubated with rat anti-ICAM-1 antibody (1:25 dilution in PBS containing 1% goat serum) at 4°C for 1 h, washed three times with PBS, and then labeled with Alexa 488-conjugated goat anti-rat IgG (1:1,000 dilution in PBS containing 1% goat serum) at 4°C for 1 h. Cells were washed in PBS and viewed under a Nikon Eclipse TE 300 fluorescence microscope at a ×100 magnification.

Western blotting. MLEC were stimulated with various concentrations of CpG ODN. Twenty minutes later, cells were scraped and analyzed by Western blotting as described (32). IκBα, phospho-IκBα, p38, and phospho-p38 mitogen-activated protein kinase (MAPK) were purchased from Cell Signaling Technology (Beverly, MA).

Statistical analysis. Each experiment was performed at least three times except where otherwise indicated, and statistical analysis was performed by the two-tailed Student’s t test by Prism software program (GraphPad Software, San Diego, CA). Data were considered significant if P < 0.05. Otherwise representative data are shown.

RESULTS

Expression of TLR9 mRNA in EC. RT-PCR analysis of RNA from MLEC, RPAEC, and RPMVEC displayed constitutive expression of transcript for TLR9, although their levels were lower than that in mouse RAW 264.7 macrophage cells. In contrast, rat pulmonary arterial smooth muscle cells showed no expression of TLR9 mRNA (Fig. 1).

ODN 1668 induces IL-8 and ICAM-1 expression in mouse but not rat lung EC. After demonstrating constitutive expression of TLR9 in mouse and rat lung EC, we then examined whether CpG DNA can induce immune response in these cells. Two different types of CpG ODN, ODN 1668 and ODN 2006, were used. They have been shown to optimally activate murine and human immune cells, respectively (7). As shown in Fig. 2A, ODN 1668 induced a strong immune response in mouse macrophages as shown by an increase in the levels of both IL-8 and ICAM-1 mRNA. MLEC also showed an increase in the mRNA levels of both IL-8 and ICAM-1 following ODN 1668 treatment, whereas non-CpG ODN treatment failed to upregulate expression of IL-8 and ICAM-1 (Fig. 2B). Surprisingly, ODN 1668 was not effective in inducing expression of IL-8 and ICAM-1 in either RPAEC (Fig. 2C) or RPMVEC (data not shown). ODN 2006, an ODN effective in activating human immune cells, was not effective in upregulating IL-8 and ICAM-1 expression in either MLEC or RPAEC (Fig. 2, B and C). Macrophages were only slightly activated by ODN 2006 (Fig. 2A).

ODN 1668-induced increases in mRNA for IL-8 and ICAM-1 in MLEC were dose dependent (Fig. 3A) and followed a characteristic time course (Fig. 3B). The increase in IL-8 and ICAM-1 mRNA expression was associated with an increase in immunoreactive IL-8 (Fig. 3C) and ICAM-1 (Fig. 3D). The latter effect, as detected by immunofluorescence, was similar to the effect of LPS.

Escherichia coli plasmid DNA is effective in inducing IL-8 expression in both MLEC and RPAEC. E. coli plasmid DNA (pEGFP-N1; Clontech, Palo Alto, CA) was purified by Endofree plasmid kit (Qiagen). MLEC and RPAEC were then treated with various concentrations of plasmid DNA, and IL-8 expression was examined 3 h later by RT-PCR. In contrast to CpG ODN, which was active in inducing IL-8 expression in
MLEC but not RPAEC, plasmid DNA was effective in up-regulating IL-8 expression in both types of cells (Fig. 4).

Chloroquine blocked CpG ODN-induced upregulation of IL-8 and ICAM-1 in MLEC. Internalization and endosomal maturation have been shown to be required for CpG DNA to activate TLR9 signaling in immune cells (17). Chloroquine, an agent that is effective in blocking endosomal maturation, significantly inhibited the CpG ODN-induced upregulation of IL-8 and ICAM-1 in MLEC (Fig. 5, A and B), suggesting similarity between MLEC and immune cells (17) with respect to CpG ODN-mediated signaling. Chloroquine had no effect on LPS-induced immune response in MLEC at the same concentration used (data not shown).

Effect of signaling inhibitors on CpG ODN-induced immune response in MLEC. CpG ODN has been shown to stimulate several signaling pathways, including extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and NF-κB in macrophages and other immune cells (4, 24, 26). Our results show that treatment of MLEC with CpG ODN induced activation of IKK and p38 MAPK. As shown in Fig. 5A, CpG ODN challenge significantly stimulated the phosphorylation of IkBα. Phosphorylation of IkBα in CpG-treated cells was associated with a concurrent decrease in the total level of IkBα at a 20-min posttreatment time point (Fig. 5A). Figure 5A also shows that CpG ODN treatment significantly activated the phosphorylation of p38 MAPK in MLEC. Accordingly, we determined the pharmacological sensitivities of these pathways that may contribute to expression of IL-8 and ICAM-1 after CpG ODN exposure in MLEC. As shown in Fig. 5B, the p38 MAPK inhibitor SB-203580 dramatically blocked the CpG ODN-induced upregulation of IL-8 and ICAM-1 mRNA. The CpG-induced expression of IL-8 and ICAM-1 was also partially inhibited by NF-κB inhibitor PDTC or SN-50. However, the MEK inhibitor PD-98059 had no effect on CpG ODN-stimulated IL-8 or ICAM-1 expression. We confirmed these results by determining changes in levels of IL-8 protein after CpG ODN exposure in MLEC (Fig. 5C). These results suggest that CpG ODN induces upregulation of IL-8 and ICAM-1 in MLEC through the activation of NF-κB and p38 MAPK signaling pathways.

Co-stimulation with LPS and CpG induces the upregulation of IL-8 and ICAM-1 in a synergic manner. The above studies clearly showed that CpG ODN was effective in inducing inflammatory response in MLEC. Because EC are likely to be exposed to both LPS and bacterial DNA concomitantly during bacterial infection, we then examined whether LPS and CpG have a synergistic effect in inducing IL-8 and ICAM-1 expression in MLEC. As shown in Fig. 6, there was no obvious increase in the levels of both IL-8 and ICAM-1 when MLEC were treated with low concentrations of either LPS or CpG ODN. However, combination of both led to a dramatic enhancement in both IL-8 and ICAM-1 response.

DISCUSSION

The innate immune system is important in host defense against invading pathogens such as bacteria and viruses. TLRs, a family of receptors, are involved in innate immune response and function by recognizing specific conserved components of microorganisms (3). Recently, TLR9 was found to be critical in the recognition of unmethylated CpG motifs in microbial DNA. Its expression is a prerequisite for the responsiveness to CpG DNA and has been shown to be expressed in many types of immune cells as well as in human colonic epithelial cells (27). In the current study, we show for the first time that rodent pulmonary EC express TLR9 (Fig. 1). CpG DNA triggers a proinflammatory response in MLEC as demonstrated by an increase in mRNA for ICAM-1 and IL-8 (Fig. 2). CpG ODN also increased immunoreactive IL-8 (Fig. 3C) and ICAM-1 (Fig. 3D).

It is interesting to note that mouse lung EC respond to both CpG ODN 1668 and endotoxin-free plasmid DNA, whereas rat pulmonary EC are activated by plasmid DNA but not ODN 1668 (Fig. 4). This might be due to the fact that ODN 1668 has a sequence that was optimized for the murine system (7). It has been shown that the immunostimulatory potency of a CpG ODN is greatly affected by the flanking sequences (16). Furthermore, the optimal sequences for activating TLR9 vary among different species (7, 12). The CpG motifs that are most active in the mouse system are poorly active in human systems and vice versa. For example, HEK-293 cells transfected with murine TLR9 are more responsive to CpG motifs optimized for activity in mice (GACGGT) than humans (GTCGTT). Similarly, cells expressing human TLR9 responded optimally to GTCGTT rather than GACGGT (7). Our data suggest that differences may exist even among closely related species such as rats and mice, with respect to the optimal sequences of CpG motifs. The mechanism for the difference in the

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CrG-INDUCED IMMUNE RESPONSE IN ENDOTHELIAL CELLS

Fig. 2. Oligodeoxynucleotide (ODN) 1668 induces IL-8 and ICAM-1 expression in mouse but not rat lung endothelial cells (EC). MLEC and RPAEC were treated with CpG and non-CpG ODN (10 μg/ml) for 3 h, and IL-8 and ICAM-1 expression in the cells was then examined by RT-PCR. A: mouse macrophages; B: MLEC; C: RPAEC. Shown in the figure are the representative data of 3 separate experiments.
Fig. 3. ODN 1668-induced increases in mRNA for IL-8 and ICAM-1 in MLEC were dose dependent and followed a characteristic time course. A: IL-8 and ICAM-1 mRNA expression in MLEC following treatment with various doses of CpG ODN. B: IL-8 and ICAM-1 mRNA expression in MLEC at different times following treatment with CpG ODN (10 μg/ml). C: ELISA analysis of IL-8 production by MLEC 24 h following treatment with various doses of CpG ODN (n = 3, *P < 0.05 compared with control). D: Immunodetection of ICAM-1 expression in MLEC following overnight treatment with CpG ODN (10 μg/ml). LPS-treated group is included as a positive control.
optimal CpG sequences among different species is not clearly understood at present. It might be possible that TLR9 of different species specifically recognizes different CpG sequences. The fact that plasmid DNA is active in both mouse and rat EC may be due to the presence of a large number of CpG motifs with different flanking sequences that are active in inducing a proinflammatory response in EC from different species, respectively. Studies are currently underway in examining how active and nonactive CpG ODNs differ in their interaction with TLR9 in cultured cells that stably express fluorescence-tagged TLR9.

CpG ODN activation has been shown to be dependent on internalization and endosomal maturation in macrophage and other immune cells. A similar observation has been made in pulmonary EC in this study (Fig. 5). Pretreatment with chloroquine, an inhibitor of vesicular acidification and endosomal maturation, dramatically inhibited the CpG ODN-induced upregulation of IL-8 and ICAM-1 expression.

The molecular mechanism by which CpG DNA activates EC has been unclear. Our preliminary studies (Western) showed that IKK and p38 were activated following treatment of MLEC with CpG ODN. Furthermore, we showed that the CpG-induced IL-8 and ICAM-1 expression was inhibited by either NF-κB inhibitor (PDTC or SN-50) or p38 inhibitor (SB-203580), suggesting that CpG DNA upregulates IL-8 and ICAM-1 expression via NF-κB- and p38 MAPK-dependent signaling pathways. A partial inhibition by NF-κB inhibitors might be due to the fact other transcriptional factors such as activator protein-1 may also be involved in the CpG-induced immune response in EC, which warrants future studies.

ERK1/2 signaling has been shown to be activated in IL-8 induction in many types of cells, and CpG DNA can induce the activation of ERK1/2 in immune cells (31, 33). Nonetheless, in rodent pulmonary EC in our study (Fig. 5), CpG ODN-induced expression of IL-8 and ICAM-1 was not affected by PD-98059, suggesting that ERK1/2 pathway is not involved. The mechanisms that are responsible for the differences in the CpG signaling among different types of cells are not clear at present and require further study. It also remains to be investigated whether vascular EC of different species differ in CpG signaling.
Simultaneous exposure to different antigenic components of bacteria, such as LPS and lipoprotein or CpG DNA and LPS, has been recognized to act synergistically to induce cytokine production in cultured immune cells and in intact animals (30). Recently, Equils et al. (9) reported that costimulation of TLR4 and TLR2 or TLR9 induced synergistic release of the Th1 cytokines IFN-γ and TNF-α. Here we also show that costimulation with CpG ODN and LPS led to synergistic increase of IL-8 and ICAM-1 expression (Fig. 6). Although different TLRs are known to induce distinct cellular and systemic responses to infection, engagement of TLR4 and TLR9 induces similar proinflammatory response. Our results are consistent with cooperation between TLR9 and TLR4 in stimulation of immune response in vascular EC.

Results from this study may have important implications in host defense and gene therapy strategies. In particular, a new role of CpG DNA and vascular endothelium in systemic bacterial infection is possible. EC injury and/or dysfunction play an important role in gram-negative sepsis (20). To date, LPS has been the major focus in studying the EC injury and/or dysfunction associated with gram-negative sepsis (6). The fact that CpG DNA is also active in inducing proinflammatory response in vascular endothelium may suggest its involvement in the development and/or progression of sepsis. This is particularly highlighted by the fact that LPS and CpG have a synergistic effect in inducing proinflammatory response in vascular EC. More studies are needed to better understand the roles of bacterial CpG DNA in systemic infection.

This study also has important implications in pulmonary endothelium gene transfer. The fact that expression plasmid can have a direct, CpG-mediated biological effect on EC not only raises a safety concern but also suggests caution in interpreting experimental data. Attention should be paid to distinguish the therapeutic gene-mediated biological effect from the CpG-mediated nonspecific effect. Strategies are currently being developed to modify the plasmid expression vectors to decrease CpG immunostimulatory activity.

In conclusion, we have demonstrated here that EC express TLR9 mRNA. Exposure to CpG DNA induced a potent proinflammatory response in mouse EC via NF-κB and p38 MAPK-dependent signaling pathways. This study suggests a possible role for CpG DNA-mediated EC immune response in the host defense system. It also has important implications in plasmid DNA-mediated pulmonary endothelium gene transfer.

REFERENCES


