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*J Immunol* 2001; 167:2847-2854; doi: 10.4049/jimmunol.167.5.2847
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A Novel Function of Phosphorothioate Oligodeoxynucleotides as Chemoattractants for Primary Macrophages

Kwan Hyuck Baek, Sang Jun Ha, and Young Chul Sung

Phosphorothioate cytosine-guanine oligodeoxynucleotides (CpG PS-ODNs) has been reported to induce Th1 immune responses against coadministered Ags more efficiently than phosphodiester CpG ODNs (CpG PO-ODNs). Here, we demonstrated that PS-ODNs, but not PO-ODNs, have a chemotactic effect on primary macrophages, which is independent of the CpG motif. In addition, the conjugation of a hexameric dG run (dG₆ run) at the 3′ terminus reduced the concentration required for the optimal chemotactic activity of PS-ODNs by ~10-fold. Endosomal maturation blockers, such as monensin and chloroquine, inhibited the chemotactic effect of PS-ODNs. The inhibition of the activities of p38 mitogen-activated protein (MAP) kinase, and extracellular signal-related kinases (ERKs) as well as phosphoinositide 3-kinase with their specific inhibitors also resulted in suppressing the chemotaxis of primary macrophages induced by PS-ODNs. These results indicate that the PS-ODN-mediated chemotaxis requires the activation of ERKs, p38 MAP kinase, and phosphoinositide 3-kinase as well as endosomal maturation. In addition, the phosphorylations of the p38 MAP kinase, ERKs, and protein kinase B, Akt, were induced by PS-ODN, which were further enhanced by the presence of both a dG₆ run and CpG motifs. Our findings suggest that the chemotactic activity of PS-ODNs may be one of the mechanisms by which PS-ODNs exhibit stronger immunomodulatory activities than PO-ODNs in vivo. The Journal of Immunology, 2001, 167: 2847–2854.

Oligodeoxynucleotides (ODNs) containing unmethylated cytosine-guanine (CpG) motifs (CpG ODNs) have been extensively studied as powerful immunomodulatory agents that are capable of activating both innate and adaptive immunity (1–6). In vitro and in vivo studies have shown that unmethylated CpG motifs in bacterial DNA and synthetic CpG ODNs can strongly stimulate macrophages and dendritic cells (DCs) to induce the production of various cytokines and the up-regulation of MHC class II and costimulatory molecules (7–10). A conversion of immature DCs to professional APCs by CpG ODNs might explain their strong adjuvant effect in promoting productive Th1 responses in vivo (11–17).

Phosphodiester ODNs (PO-ODNs) containing CpG motifs have shown to mimic the effects of native bacterial DNA in vitro. However, their short half-life (18) and poor immunogenicity in vivo (17) seemed to circumscribe their use in clinical applications. For these reasons, phosphorothioate ODNs (PS-ODNs) have been preferred for use in most in vivo studies. For example, PS-ODNs have been reported to be effective adjuvants in vaccinations (11–14, 19) and potent agents in immunotherapy of inflammatory as well as allergic diseases (20–22). Recently, we showed that PS-ODNs induced Th1 immune responses in vivo more efficiently than PO-ODNs conjugated to hexameric deoxyribo-guanosine (dG) residues (dG₆ runs) at their 3′ terminus, although both of them induced a similar level of cytokine production from APCs (17). In addition, the dG run was reported to increase receptor-mediated endocytosis of ODNs and to confer serum nuclease resistance by forming a tetraplex structure (17, 23). Moreover, Sester et al. (24) have demonstrated that the PS backbone itself has immunomodulatory effects on the responses of macrophages to CpG ODNs. Thus, it was of interest to determine the factors contributing to the powerful immunomodulatory effect of PS-ODNs in vivo.

It has been reported that p38 mitogen-activated protein (MAP) kinase and extracellular signal-related kinases (ERKs or p44/42 MAP kinase) are involved in the chemotaxis of immune cells such as neutrophils and eosinophils (25, 26). CpG ODNs were known to induce the phosphorylation of these kinases in primary macrophages, bone marrow-derived DCs, B cell lines, and monocyte-like cell lines (27–29). In addition, PS backbone was reported to modulate the phosphorylation of ERKs by CpG motifs in primary macrophages (24). In particular, PS-ODNs, but not PO-ODNs, induced the proliferation of human B cells (30–32). It is noteworthy that phosphoinositide 3-kinase (PI3K) signaling cascades are involved in chemotaxis as well as proliferation of neutrophils and macrophages elicited by fMLP (33–35). These observations led us to suggest that signal transduction cascades induced by PS-ODNs might be associated with those involved in chemotactic migration and that PS-ODNs could induce chemotactic migration of APCs such as macrophages.

In this study, we found that PS-ODNs, independent of CpG motifs, act as chemoattractants on primary macrophages in vitro and that the activation of p38 MAP kinase, ERKs, and PI3K plays an important role in the chemotactic activity. Based on our data, we suggest that the chemotactic effect by the PS backbone itself may be one of the mechanisms by which PS-ODNs induce stronger immune activation than PO-ODNs in vivo.
Materials and Methods

Animals

Eight- to 10-wk-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and used for the isolation of peritoneal macrophages. These animals were maintained in a positive pressure facility (one-way flow) and fed autoclaved food and water.

ODNs, reagents, and Abs

All CpG and non-CpG ODNs with PO and PS backbones were purchased from GenoTech (Taejon, Korea). Poly(dG) and M21 were modified by tagging the 5’ terminus with fluorescein (f-ODNs). LPS ODNs content of ODN was <1 ng LPS/mg ODN as measured with Limulus amebocyte assay reagents obtained from Sigma (St. Louis, MO). The maximum concentration of ODN used in the chemotaxis assay was 100 μg/ml, which contained LPS <0.1 ng/ml. General reagents used were purchased from Sigma unless otherwise indicated. SB202474, SB203580, PD90859, and Ly294002 were purchased from New England Biolabs (Beverly, MA). Rabbit Abs specific against p38 MAP kinase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit Abs against ERKs and protein kinase B, Akt, were purchased from New England Biolabs (Beverly, MA). Rabbit Abs specific against Thr32/Tyr36-phosphorylated p38 MAP kinase, Thr202/Tyr204-phosphorylated ERKs, and Ser473-phosphorylated Akt were purchased from New England Biolabs. Abs of HRP-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology.

Preparation of marine peritoneal macrophages

Resting peritoneal macrophages were harvested as previously described (36) by washing them out of the peritoneal cavity of BALB/c mice with RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 50 μM 2-ME, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and seeding in a tissue culture dish. After incubation for 9 h, nonadherent cells were removed by washing extensively with PBS. Remaining adherent cells were used in the following experiments.

Chemotaxis assay

Chemotaxis induced by ODNs was assayed using a modification of the 96-well microchemotaxis assay as described previously (37). ODNs, iMLP as a positive control, and BSA (Life Technologies) as a negative control were each diluted into HBSS medium containing 0.2% BSA to the various concentrations. The specimens were added to the lower wells of a 96-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). The carbon membrane purchased from Neuroprobe (Cabin John, MD) for 15 min at 4°C and further incubated with 1 μM f-ODNs for 30 min at 4°C. After thoroughly washing the cells with PBS, the mean fluorescent intensity (MFI) emanating from f-ODNs bound to the cells was measured using FACSscan and CellQuest software. The MFI was calculated for live cells, after subtracting the dead population of cells determined by propidium iodide staining.

Preparation of whole cell lysates and immunoblot analysis

Peritoneal macrophages (2 × 10^6 cells/ml) were treated with medium, ODNs, or LPS for 24 h. The culture supernatants were analyzed by a commercial ELISA kit (Genzyme, Cambridge, MA) for the detection of IL-12p70.

Cytokine ELISA

Peritoneal macrophages (2 × 10^6 cells/ml) were treated with medium, ODNs, or LPS for 24 h. The culture supernatants were analyzed by a commercial ELISA kit (Genzyme, Cambridge, MA) for the detection of IL-12p70.

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Preparation of whole cell lysates and immunoblot analysis

Peritoneal macrophages (2 × 10^6 cells/ml) were treated with medium, LPS (5 μg/ml), CpG, or non-CpG PS-ODNs. Cells were harvested at the indicated time points, and whole cell lysates were prepared as described previously (38). To quantify the phosphorylation of ERKs, p38 MAP kinas, and protein kinase B, Akt, equal amounts of whole-cell lysates (70 μg/lane) were subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE). Then Western blots were immunoanalyzed as described previously (38) using specific Abs against the phosphorylated form of each of the proteins. To confirm that the same amount of cellular protein had been loaded in each lane, the primary Ab/secondary Ab complex was removed by incubating the blot in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50°C. The blots were then subjected to autoradiography to confirm that the Ab signal had been removed. After this procedure, the blots were reprobed with the specific Abs against total p38 MAP kinase, ERKs, and Akt.

Table I. Sequences of ODNs used in this studya

<table>
<thead>
<tr>
<th>Name</th>
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</tr>
<tr>
<td>1826DG</td>
<td>TCCATGACGTTGCCTGAGCTT</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>M21E</td>
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a Bold, phosphorothioate linkage; underlined, CpG dimucleotides.
Results

ODNs with PS, but not PO, backbone induce the migration of primary macrophages, which is further enhanced by the presence of a hexameric dG run

To investigate the effects of ODNs on the chemotaxis of primary macrophages, we isolated mouse peritoneal macrophages and performed chemotaxis assays with CpG and non-CpG ODNs that had either PS (1826T) or PO (1826D and 1826DGC) backbones. The ODN 1826T had been reported to stimulate murine leukocytes, including macrophages and DCs, and B lymphocytes (39). In addition, we recently reported that the conjugation of a dG run at the 3’ terminus of PO-ODNs induces receptor-mediated endocytosis of ODNs through their binding to a receptor with a scavenger receptor type A (SR-A) ligand specificity (17). Therefore, we tested the chemotactic activity of a modified PO-ODN (M21) that was conjugated with a dG6 run at the 3’ terminus of 1826D and its non-CpG form (M21GC) (Table I). As expected, M21, but not M21GC, significantly induced the expression of CD86 and the production of IL-12, which is comparable to 1826T (Fig. 1, B and C). However, all PO-ODNs, including M21, failed to induce the migration of primary macrophages at even high concentration (20 μM) (Fig. 1A). In contrast, the PS-ODN, 1826T, was shown to exhibit chemotactic activity in a dose-dependent manner (Fig. 1A). Next, we were interested in determining whether the CpG motifs and the PS backbone of 1826T worked synergistically and whether other specific flanking sequences were involved in the chemotaxis of macrophages induced by PS-ODNs. To address these questions, we compared the chemotactic activity induced by SdC28 and SdG28 as well as the various derivatives of 1826T and p19T. Interestingly, there was no significant difference (p > 0.05) in the chemotactic activity between CpG PS-ODNs and non-CpG PS-ODNs (1826T vs 1826TGC, M21T vs M21TGC, and M16T vs M16TGC) (Fig. 2A). In addition, both SdG28 and SdC28 also induced the chemotactic migration of macrophages comparable to other PS-ODNs. In particular, the introduction of two PS linkages into the terminus of PO-ODN, M21E, endowed the ODN with a chemotactic effect on the treated macrophages. Moreover, M21T and 1826T induced the chemotactic migration of primary macrophages in a bell-shaped concentration-response curve, which is a typical feature of chemoattractants (Fig. 2B). It is worthwhile to note that PS-ODNs carrying a 3’ dG6 run induced the optimal migration of macrophages at a 10-fold lower concentration than PS-ODNs without a 3’ dG6 run (Fig. 2, A and B), indicating that the presence of a 3’ dG6 run facilitated the chemotactic migration of primary macrophages. To further investigate whether the conjugation of a dG run at the 3’ terminus of PS-ODNs affects their binding to SR-A on macrophages, which would increase their uptake into the cells, we performed competitive binding assays (Fig. 3, A and B). As ligands, we used f-poly(dG) and f-M21, which bind to receptors with a SR-A ligand specificity (17). 1826T, M21T, and M16T were used as competitors. The binding of f-poly(dG) and f-M21 to macrophages was significantly impaired by treatment of the cells with M21T and M16T in a dose-dependent manner. In contrast, 1826T that does not carry a 3’ dG6 run did not compete with f-poly(dG) and f-M21 for binding to macrophages even at a high concentration (5 μM). Taken together, these data imply that the PS backbone of PS-ODNs is able to elicit the migration of primary macrophages through a CpG motifs-independent mechanism and that a dG6 run conjugated to the 3’ terminus of PS-ODNs can further enhance the chemotactic activity of macrophages, presumably through their binding to a receptor with a SR-A ligand specificity.
maturation, we examined the effect of endosomal acidification blockers, such as chloroquine and monensin, on the chemotaxis of primary macrophages stimulated by M21T (Fig. 4A). In a previous study, $2-5 \mu g/ml$ and $20 \mu M$ concentrations of chloroquine and monensin, respectively, were shown to inhibit effectively the endosomal maturation-dependent immunomodulatory activity of CpG ODNs (40). When these ranges of inhibitor concentrations were used in the chemotaxis assay, chloroquine appeared to block the M21T-mediated migration of primary macrophages in a dose-dependent manner, whereas monensin completely blocked the migration of macrophages. Similar results were observed in assays for which 1826T and M21TGC were used (data not shown). These data suggest that the PS backbone-mediated signaling pathway involved in the chemotaxis of macrophages may be preceded by endosomal maturation.

Cellular MAP kinases have been shown to play an important role in the chemoattractant-mediated migration of neutrophils and eosinophils (25, 26, 41). In addition, neutrophils and peritoneal macrophages obtained from PI3K-deficient mice have shown the reduced migration in response to a wide range of chemotactic stimuli (33–35). To investigate whether cellular MAP kinases and PI3K were involved in the PS-ODN-mediated chemotaxis, peritoneal macrophages were isolated and preincubated with specific inhibitors for p38 MAP kinase (SB203580), ERKs (PD90859), and PI3K (wortmannin and Ly294002) before undergoing the chemotaxis assay (Fig. 4B). It has been reported that $1-10 \mu M$ SB203580, $10-100 \mu M$ PD90859, $50-100 \mu M$ wortmannin, and $20-50 \mu M$ Ly294002 effectively inhibited each kinase-dependent chemotactic migration of neutrophils and eosinophils (35, 41). As expected, SB202474, a negative analog of SB203580, failed to have any effect on the chemotactic migration of primary macrophages elicited by M21T even at a high concentration ($50 \mu M$). However, SB203580 and PD90859 reduced the number of primary macrophages migrating toward M21T in a dose-dependent manner. The migration of primary macrophages was significantly reduced by $\sim 60\%$ after treatment of the macrophages with $1 \mu M$ chloroquine or $10 \mu M$ PD90859.

![FIGURE 2. The effect of CpG motifs and a dG₆ run on the chemotaxis of macrophages elicited by PS-ODNs. Various PS-ODNs (A) or 1826T and M21T (B) were added to the lower wells of a 96-well chemotaxis chamber in various concentrations as indicated. BSA was used as a negative control at 1, 10, and 100 \mu g/ml. The chemotaxis assays were then performed as described in Materials and Methods. Data represent the number of cells migrated across the filter, which are averages of triplicate readings from three independent experiments.](http://www.jimmunol.org/)

![FIGURE 3. PS-ODNs with a 3’ dG₆ run impede the binding of f-ODNs to receptors with a SR-A ligand specificity in a dose-dependent manner. Peritoneal macrophages ($10^5$) were preincubated with 1826T, M21T, or M16T (1 and 5 \mu M) for 15 min at 4°C, and then further incubated with 1 \mu M f-poly(dG) (A) or f-M21 (B) for 30 min at 4°C. MFI of bound f-ODNs was determined by flow cytometry. Data represent relative percentages of the binding determined for f-ODN alone (set as 100%). All experiments were repeated three times with similar results. Data are means ± SEM of three independent experiments each performed in triplicate.](http://www.jimmunol.org/)
SB203580 and completely blocked upon treatment with 50 μM SB203580. In addition, PD90859 slightly reduced the number of migrated primary macrophages at a 10 μM concentration, but significantly reduced it at 50–100 μM. Wortmannin and Ly294002 also blocked the chemotactic migration of macrophages toward M21T in a dose-dependent manner, implying that the PI3K activity is also critical for the PS-ODN-mediated chemotactic migration of macrophages. The chemotaxis of macrophages elicited by other PS-ODNs such as 1826T, 1826TGC, and M21TGC was also blocked in a similar pattern by these inhibitors (data not shown). However, we exclude the possibility that the chemotactic recruitment of peritoneal macrophages might be due to the toxicity of specific inhibitors used in the chemotaxis assay, because there were no significant differences in the viability and little detectable change in morphology of peritoneal macrophages under these experimental conditions (data not shown). In this respect, our observations suggest that p38 MAP kinase, ERKs, and PI3K are closely associated with the PS-ODN-mediated signal transduction pathway required for the chemotaxis of macrophages.

**PS-ODNs induce the phosphorylation of ERK-1/2 and p38 MAP kinase in primary macrophages**

Because CpG PS-ODNs, but not non-CpG PS-ODNs, were reported to induce the activation of ERKs in primary macrophages and the phosphorylation of p38 MAP kinase in monocyte-derived cell lines, B cell lines, and bone marrow-derived DCs (27, 28), our results that the activation of kinases was CpG-independent seemed to be inconsistent with previous observations (Fig. 4B). To evaluate the kinetics and levels of the phosphorylations of these kinases in primary macrophages stimulated with non-CpG PS-ODNs (M21TGC and 1826TGC), primary macrophages were treated with M21TGC and 1826TGC at concentrations of 2 and 20 μM, respectively. M21TGC (2 μM) and 20 μM 1826TGC were shown to be concentrations required for the optimal recruitment of primary macrophages (Fig. 2A). As positive controls, CpG PS-ODNs (M21T and 1826T) were also used. As expected, all non-CpG PS-ODNs as well as CpG PS-ODNs induced the phosphorylation of ERK-1/2 and p38 MAP kinase within 30 min (Fig. 5, A and B). It is likely that 1826T and 1826TGC induce slightly higher phosphorylations of p38 MAP kinase at 20 μM than those at 2 μM (Fig. 5B), which are reminiscent of the earlier result that 1826T and 1826TGC induce the optimal chemotactic migration of macrophages at 20 μM (Fig. 2). Of interest, the kinetics of ERK-1/2 phosphorylation by 1826T and 1826TGC were slightly slower than those by M21T and M21TGC. Similarly, stimulation of peritoneal macrophages with M21T and M21TGC led to more rapid induction of the phosphorylation of p38 MAP kinase (peaked at 7 min) than those with 1826T and 1826TGC (peaked at 30 min), suggesting that a 3' dG run may accelerate the phosphorylation of ERK-1/2 and p38 MAP kinase. However, it is likely that there is little correlation between the chemotactic activity of PS-ODNs and the kinetics of the phosphorylation of these MAP kinases, because the chemotactic migration of macrophages by 20 μM 1826T is comparable to that by 2 μM M21T as shown in Fig. 2B. The phosphorylation of ERK-1/2 by M21T was demonstrated to be slightly higher than that by M21TGC after 30 min. In addition, the phosphorylation of p38 MAP kinase by M21TGC declined after 15 min, but that by M21T was retained up to 30 min. In contrast, there was no significant difference between 1826T and 1826TGC in inducing the phosphorylation of ERK-1/2 and p38 MAP kinase, at least within 30 min. These results indicate that the effect of CpG motifs on the phosphorylation of these MAP kinases in macrophages is dependent on a 3' dG run. However, the increased phosphorylation of this MAP kinase by CpG motif did not have significant effects on the chemotactic activity of M21T compared with that of M21TGC (Fig. 2A).

**PS-ODNs induce the activation of PI3K/Akt signaling cascades**

It has been reported that protein kinase B, Akt, is involved in a downstream signaling pathway for the chemotactic recruitment of...
Here we demonstrated that the activation of PI3K is critical for the chemotaxis of peritoneal macrophages by PS-ODNs, suggesting that the activation of PI3K/Akt signaling cascades induced by PS-ODNs may be involved in the chemotactic recruitment of primary macrophages by PS-ODNs. As expected, all PS-ODNs tested induced the phosphorylation of Akt in primary macrophages (Fig. 5C). As the cases of ERK-1/2 and p38 MAP kinase, the phosphorylation of Akt by M21T and M21TGC occurs at the earlier time point than that by 1826T and 1826TGC, suggesting that a 3’ dG_a run may also accelerate the activation of PI3K. In addition, the phosphorylation of Akt by M21T was slightly faster and higher than that by M21TGC for up to 30 min, whereas there was no significant difference between 1826T and 1826TGC in inducing the phosphorylation of Akt. These observations suggest that the effect of CpG motifs on the phosphorylation of Akt by PS-ODNs is dependent on a 3’ dG_a run. In addition, the kinetics and level of Akt phosphorylation induced by PS-ODNs were shown not to directly correlate with the chemotaxis of peritoneal macrophages (Figs. 2 and 5C).

Discussion

There are many reports that sulfite is toxic to the lung and causes allergic reactions, which are characterized by a prominent neutrophil influx (42–45). In addition, sulfite was reported to be a strong reducing agent and to have immunomodulatory effects on human alveolar macrophages in vitro (46). Thus, it is possible that sulfite contained in chemical compounds is an environmental risk factor and also acts as a danger signal to cells of the innate immune system. The PS-ODNs have sulfur that is substituted for one of the nonbridging oxygens in the PO backbone and have been used in neutrophils via PI3K activation (35). Here we demonstrated that the activation of PI3K is critical for the chemotaxis of peritoneal macrophages by PS-ODNs, suggesting that the activation of PI3K/Akt signaling cascades induced by PS-ODNs may be involved in the chemotactic recruitment of primary macrophages by PS-ODNs. As expected, all PS-ODNs tested induced the phosphorylation of Akt in primary macrophages (Fig. 5C). As the cases of ERK-1/2 and p38 MAP kinase, the phosphorylation of Akt by M21T and M21TGC occurs at the earlier time point than that by 1826T and 1826TGC, suggesting that a 3’ dG_a run may also accelerate the activation of PI3K. In addition, the phosphorylation of Akt by M21T was slightly faster and higher than that by M21TGC for up to 30 min, whereas there was no significant difference between 1826T and 1826TGC in inducing the phosphorylation of Akt. These observations suggest that the effect of CpG motifs on the phosphorylation of Akt by PS-ODNs is dependent on a 3’ dG_a run. In addition, the kinetics and level of Akt phosphorylation induced by PS-ODNs were shown not to directly correlate with the chemotaxis of peritoneal macrophages (Figs. 2 and 5C).

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most applications of CpG DNA to immunotherapy and antisense therapy because of their relative stability. Based on these facts, sulfur contained in the backbone of CpG PS-ODN by itself may function as an activating signal, which is distinguished from those of CpG motifs. In this report, we clearly demonstrated that PS-ODNs, but not PO-ODNs, induce the chemotactic recruitment of primary macrophages. Because the PS-ODNs exhibited their chemotactic effect on primary macrophages in the absence of CpG motifs, our results suggest that the PS backbone itself may have immunomodulatory properties via a signaling pathway that is separated from the CpG motif-mediated signaling. Our results partially agree with a recent report that the PS backbone appeared to have both enhancing and inhibitory effects on macrophage responses such as the production of NO and the activation of IL-12 promoter (24). It is noteworthy that the tagging of the 3’ terminus of PS-ODNs with a dG, run was found to reduce the concentration required for the optimal recruitment of macrophages, presumably through the binding of the ODNs to a receptor with a SR-A ligand specificity. This can be explained as follows: First, the binding of PS-ODNs carrying a dG, run to the SR increases the amount of internalized PS-ODNs into cells via the receptor-mediated endocytosis, which could augment the PS backbone-mediated signaling. Second, it was reported that guanosine-rich ODNs induced the proliferation of macrophage progenitor cells (47) and that the SR clustering by specific ligands induced the activation of ERKs and p38 MAP kinase (48). It is likely that these MAP kinases are associated with the chemotaxis of macrophages elicited by PS-ODNs. Thus, the ligation of a dG, run to the SR itself might produce an additive signal for the chemotaxis of macrophages by PS-ODNs. However, M21E, PO-ODNs containing a dG, run, failed to induce the chemotactic migration of peritoneal macrophages, indicating that the dG, run alone is not sufficient to elicit chemotaxis.

We demonstrated that the activation of the signal transduction pathway by the PS backbone must be preceded by endosomal maturation. Endosomal maturation is known to be required for the activation of APCs by CpG ODNs to induce cytokine production (29). In this regard, these results suggest that common intracellular factors appeared to be used for both the PS backbone- and CpG motifs-mediated signal pathways. In addition, the inhibition of cellular MAP kinases such as p38 MAP kinase and ERKs was found to abolish the chemotactic effect of PS-ODNs. Moreover, the phosphorylation of these MAP kinases was demonstrated to be significantly induced in primary macrophages stimulated by non-CpG PS-ODNs. However, M21E, PO-ODNs containing a dG, run, failed to induce the chemotactic migration of peritoneal macrophages, indicating that the dG, run alone is not sufficient to elicit chemotaxis.

In this study, we demonstrated that PI3K was activated by the PS backbone-mediated signal, which was further enhanced by the CpG motif-mediated signaling in the presence of a 3’ dG, run (M21T vs M21TGC). This result indicates that PI3K is synergistically activated in primary macrophages by the PS backbone and CpG motifs. PI3K plays an important role in the proliferation of B cells and macrophages (49–51). Our observations support the previous results that CpG PS-ODNs, but not CpG PO-ODNs and non-CpG PS-ODNs, promoted human B cell proliferation (30–32). In addition, we recently observed that CpG ODNs stimulated the proliferation of murine B cells in proportion to the number of PS linkages within CpG ODNs (data not shown).

Even though these kinases are essential for the chemotactic recruitment of peritoneal macrophages, there is no direct correlation between the chemotactic activity of PS-ODNs and the kinetics of the phosphorylation of cellular MAP kinases and PI3K. Therefore, it is possible that undefined cellular factors might play key roles in the rate-limiting step of the chemotactic recruitment of macrophages by PS-ODNs.

In general, the observation that PS-ODNs exerted stronger immunomodulatory effects than PO-ODNs has been explained by the relative stability of PS-ODNs in vivo (52, 53). In this study, we demonstrated that PS-ODNs function as chemoattractants for primary macrophages. Thus, it is possible that PS-ODNs recruit APCs at the initial step of the induction of immune responses in vivo. In other words, macrophages are recruited to the injection site by the chemotactic signals emanating from the PS backbone, and then further stimulated by signals coming from the CpG motifs, followed by the production of proinflammatory cytokines for the additional recruitment and the activation of immune cells. In partial agreement with this suggestion, it was recently reported that non-CpG PS-ODN had an adjuvant effect due to the immunomodulatory effect of the PS backbone when administered at mucosal sites (54). Because of the stability and the chemotactic activity of the PS backbone, PS-ODNs would have advantages over PO-ODNs in clinical and immunotherapeutic applications.

Acknowledgments

We thank Dr. C. W. Lee and Dr. S. W. Lee for reviewing this manuscript and S. Y. Choi for excellent technical assistance. We also thank S. C. Lee and S. I. Park for animal care.

References


