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Cellular uptake and activation characteristics of naked plasmid DNA and its cationic liposome complex in human macrophages

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Abstract

Plasmid DNA (pDNA) is an important macromolecular therapeutic agent suitable for DNA-based therapies, such as non-viral gene therapy and DNA vaccination. Unmethylated CpG motifs abundant in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response, which inhibits transgene expression, while modulating immunological consequences following vaccination. We studied cellular uptake and activation characteristics of naked pDNA and its cationic liposome complex in human macrophage-like cells. The present study has demonstrated that naked pDNA was recognized by human macrophage-like cells via specific mechanisms for polyanions. Moreover, it has shown that pDNA complexed with cationic liposomes activates human macrophage-like cells to induce the production of tumor necrosis factor-α (TNF-α) in a CpG motif-independent manner, while any types of naked DNA could not induce TNF-α production from these cells, regardless of the presence of CpG motifs in pDNA or oligonucleotide (ODN). These findings form an important basis for DNA-based therapies including gene therapy and DNA vaccination.

Keywords: Macrophages; Plasmid DNA; CpG motif; Cationic liposome; Tumor necrosis factor-α

1. Introduction

Plasmid DNA (pDNA) has become an important macromolecular therapeutic agent suitable for DNA-based therapies, such as non-viral gene therapy and DNA vaccination (Leitner et al., 1999; Nishikawa and Huang, 2001). However, unmethylated CpG motifs in pDNA or bacterial DNA, but not in vertebrate DNA, are recognized by immune cells as a danger signal (Krieg, 2002). When macrophages or dendritic cells (DCs) take up CpG DNA, inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-12 are secreted. These cytokines significantly influence DNA-based therapies in different ways. In gene therapy, the cytokine production generally seems inappropriate because these inflammatory cytokines sig-

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nificantly reduce transgene expression of therapeutic proteins in target cells through their direct cytotoxic and promoter attenuation effects (Ghazizadeh et al., 1997; Qin et al., 1997). On the other hand, these inflammatory cytokines are essential for the efficacy of DNA vaccination because these cytokines can enhance the immune responses and the balance of these cytokines profoundly affects the nature of these responses (Leitner et al., 1999; Krieg and Kline, 2000).

CpG motifs in bacterial DNA or synthetic ODNs are recognized by Toll-like receptor 9 (TLR9) and trigger an immune cascade, resulting in improved antigen uptake and presentation by antigen-presenting cells and the secretion of polyreactive Ig, chemokines and cytokines by B cells, natural killer (NK) cells, DCs and monocytes. TLR9 is abundantly expressed in immunocompetent cells in mice, but the receptor is expressed only in primarily B cells and plasmacytoid dendritic cells (pDCs) in humans (Hornung et al., 2002). In addition, some CpG ODN can induce cell type-specific immune responses. For example, CpG-A ODN produces marked induction of interferon-α (IFN-α) production from pDCs and CpG-B ODN can prominently induce the proliferation of B cells. Moreover, some ODNs containing non-CpG sequences are able to activate these cells (Bauer et al., 1999; Pasquini et al., 1999; Vollmer et al., 2002). Thus, there seems to be various dangerous sequences and, perhaps, various molecules that could recognize those signals. These possibilities could lead to problems involving the application of pDNA because bacterial DNA and pDNA contain various CpG sequences. However, most of the earlier studies have examined the effects of CpG DNA using single stranded ODN containing ∼20 phosphorothioate-stabilized nucleotides and the effects of CpG DNA in bacterial DNA or pDNA are poorly understood.

We have investigated the in vivo disposition characteristics of naked pDNA in mice and found that liver non-parenchymal, probably, Kupffer (liver resident macrophages) cells play an important role (Yoshida et al., 1996; Takagi et al., 1998). Further, in vitro studies using cultured mouse peritoneal macrophages have suggested that a specific receptor, like the class A scavenger receptor, may be involved in the endocytic uptake of pDNA by macrophages (Takagi et al., 1998). We also examined the pDNA uptake by the murine DC cell-line, DC2.4 cells, and found that DCs take up pDNA by a specific mechanism more efficiently and rapidly than macrophages (Yoshinaga et al., 2002). Moreover, we previously demonstrated that DNA complexed with cationic liposomes could activate murine peritoneal macrophages in a CpG-independent manner, while naked pDNA could not (Yasuda et al., 2002). Our recent study has demonstrated that the macrophage activation by DNA/cationic liposome complex requires endosomal acidification and both TLR9-dependent and -independent pathways (Yasuda et al., 2005).

Thus, the immune activation mediated by CpG-motif is well characterized in mice, but there is insufficient information about the effects of CpG on human immune cells. A better understanding of the effects of pDNA in humans is required in order to apply the knowledge obtained in mouse studies to human therapeutic strategies. In the present study, the uptake of pDNA by human macrophage-like cells is examined as well as the activation of human macrophages stimulated with DNA in both naked and complexed form. These findings will be an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). pCMV-Luc encoding firefly luciferase gene constructed previously was used as a model pDNA. Calf thymus DNA (CTDNA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). LipofectAMINE 2000 (LA) and Opti-MEM were purchased from Lifetechnologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). All other chemicals used were of the highest purity available.

2.2. Cell cultures

Human monocytic cell-lines, U937 cells and THP-1 cells were obtained from the Cell Resource

Center for Biomedical Research, Tohoku University, and maintained in RPMI 1640 supplemented with 10% FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL). For experiments, cells were plated on a 24-well culture plate at a density of 1×10^6 cells/mL in the presence of 100 ng/mL PMA at 37 °C in 5% CO_2–95% air for 48 h, to differentiate to macrophage-like cells.

2.3. Plasmid DNA

For cellular association experiments, pCMV-Luc was radiolabeled using [α-^{32}P]dCTP by nick translation (Rigby et al., 1977). For synthesis of methylated pDNA, cytosine residues in CpG sequences of pDNA were methylated by SssI methylase (New England Biolabs, Beverly, MA, USA). The methylated pDNA was tested for digestion with *Hpa*II (Takara, Kyoto, Japan) to confirm methylation. pDNA mobility was analyzed by 1% agarose gel electrophoresis. DNA/LA complexes were prepared according to manufacturer's instructions.

2.4. Oligodeoxynucleotides

Phosphorothioate ODN were purchased from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The sequences of CpG S-ODN 2006 are 5′-TCGTCG-TTTTGTCGTTTTGTCGTT-3′, a proven activator of human immune cells as previously described (Kerkmann et al., 2003). Phosphorothioate non-CpG ODN 2006GC (5′-TGCTGCTTTTGTGCTTTTGTG-CTT-3′) was used as a control.

2.5. Purification of DNA

To minimize the activation by contaminated lipopolysaccharide (LPS), DNA samples were used following thorough purification with Triton X-114, a non-ionic detergent. Extraction of endotoxin from pDNA, methylated pDNA, and CTDNA samples was performed according to previously published methods (Cotten et al., 1994; Hartmann and Krieg, 1999) with slight modifications as previously described (Yasuda et al., 2002). DNA samples were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. DNA (10 mg) was diluted with 20 mL pyrogen-free water, and then 200 μL Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55 °C. Subsequently, the solution was centrifuged for 20 min at 25 °C, 600 × *g*. The upper phase was transferred to a new tube, 200 μL Triton X-114 was added, and the previous steps were repeated three or more times. The activity of LPS was measured by limulus amebocyte lysate (LAL) assay using the Limulus *F*-Single Test kit (Wako, Tokyo, Japan). After Triton X-114 extraction, the endotoxin levels of DNA samples could no longer be determined by LAL assay, i.e., 1 μg/mL DNA contained less than 0.001 EU/mL. Without extraction of endotoxin by Triton X-114, 100 μg/mL naked pDNA contained 1–5 EU/mL endotoxin.

2.6. Cellular association experiments

Cells were washed with 0.5 mL HBSS without phenol red and 0.5 mL HBSS containing 0.1 μg/mL naked [^{32}P]pDNA or pDNA/LA complex was added. After incubation at 37 or 4 °C for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1 mL 0.3 M NaOH with 0.1% Triton X-100. Aliquots of the cell lysate were taken for the determination of ^{32}P radioactivity using an LAS-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content was measured using the modified Lowry method with bovine serum albumin as a standard. To examine the competition in binding, unlabeled macromolecules, such as polyinosinic acid (poly[I], M.W. 103.3 kDa), polycytidylic acid (poly[C], M.W. 99.5 kDa), dextran (M.W. 70 kDa) and dextran sulfate (M.W. 150 kDa) were added to the incubation wells concomitantly with [^{32}P]pDNA.

2.7. TCA precipitation experiments

After the cellular association experiments, the medium and cell lysates containing radioactivity derived from [^{32}P]pDNA were subjected to trichloroacetic acid (TCA) precipitation experiments to assess the degradation of pDNA by human macrophage-like cells as previously described (Yoshinaga et al., 2002).

2.8. Confocal microscopy

pCMV-Luc was labeled with fluorescein using a Fasttag FL labeling kit according to the manufacture's instructions (Vector Laboratories, Burlinghame, CA, USA). Cells were washed three times and incubated with medium containing fluorescein–pDNA or fluorescein–pDNA/LA complex. After an 8 h incubation at 4 or 37 °C, the cells were washed four times and fixed with 1% paraformaldehyde for 10 min. The cells were then scanned by confocal microscopy (MRC-1024, BIO-RAD, CA, USA).

2.9. Cytokine secretion

PMA-treated U937 cells were washed with 0.5 mL RPMI 1640 before stimulation. Naked DNA and DNA/LA complex were diluted in 0.5 mL Opti-MEM. The cells were incubated with the DNA-containing solution continuously for 8 or 24 h. In the case of DNA/LA complexes, cells were incubated for 2 h with the solution containing the complexes and then the solution was removed and the cells were incubated with Opti-MEM continuously for specified periods up to 24 h. After incubation, the conditioned medium was collected to assess cytokines and kept at −80 °C. The levels of TNF-α in the conditioned medium were quantitated by human TNF-α ELISA set (eBioscience, San Diego, CA) or OptEIA mouse TNF-α set (BD PharMingen, San Diego, CA), following the manufacturer's instructions.

3. Results

3.1. Cellular association of ^{32}P or fluorescein-labeled pDNA with naked or complexed form in human macrophage-like cells

A time-dependent increase in the cellular association of naked [^{32}P]pDNA with U937 cells was observed at 37 °C. The cellular association significantly decreased at 4 °C (Fig. 1A), where only binding to the cellular surface occurred. On the other hand, when pDNA was complexed with cationic liposomes, the amount of pDNA binding to the cell surface increased compared with naked pDNA (Fig. 1B). However, the degree of total cellular association at 37 °C was similar to that at 4 °C. This tendency was also observed in other human macrophage-like cells, PMA-treated THP-1 cells (Fig. 2). The degradation of pDNA by human macrophage-like cells was investigated by the TCA precipitation method, which can detect degraded DNA smaller than about 16mer. In both PMA-treated U937 cells and THP-1 cells, little of the pDNA was fragmented to units smaller than 16mer (data not shown).

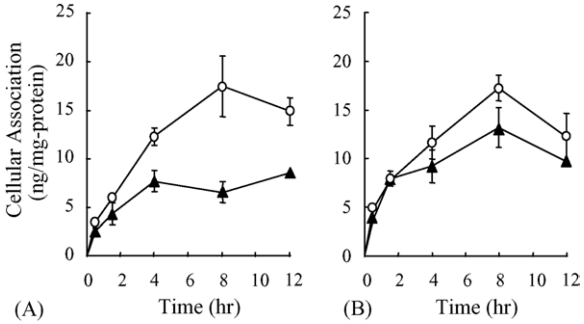

Fig. 1. Cellular association time-courses of naked [^{32}P]pDNA (A) or [^{32}P] pDNA/cationic liposome complex (B) in U937 cells. These cells were incubated with [^{32}P]pDNA (0.1 μg/mL) or [^{32}P]pDNA/LA complex (0.1 μg/mL: 0.2 μg/mL) at 37 °C (open circle) or 4 °C (closed triangle). Each point represents the mean ± S.D. (n = 3).

In order to examine the intracellular localization of pDNA, the cellular association of fluorescein-labeled pDNA by U937 cells was investigated using confocal microscopy (Fig. 3). Localization of both naked

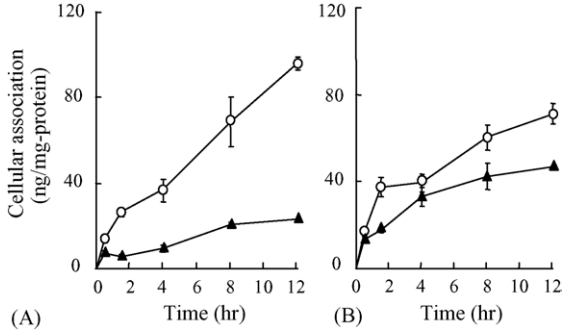

Fig. 2. Cellular association time-courses of naked [^{32}P]pDNA (A) or [^{32}P] pDNA/cationic liposome complex (B) in THP-1 cells. These cells were incubated with [^{32}P]pDNA (0.1 μg/mL) or [^{32}P]pDNA/LA complex (0.1 μg/mL: 0.2 μg/mL) at 37 °C (open circle) or 4 °C (closed triangle). Each point represents the mean ± S.D. (n = 3).

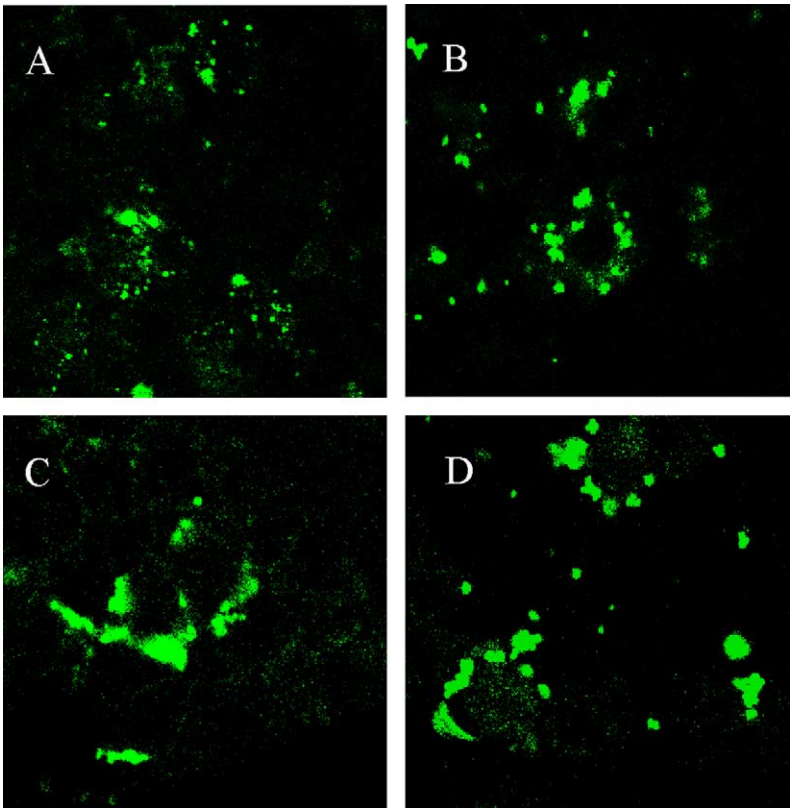

Fig. 3. Intracellular localization of naked fluorescein–pDNA or fluorescein–pDNA/LA complex in U937 Cells. The cells were incubated with naked fluorescein–pDNA (5 μg/well, A and C) or fluorescein–pDNA/LA complex (2.5 μg: 5 mL/well, B and D) for 8 h at 37 °C (A and B) or 4 °C (C and D).

and complexed pDNA at 4 °C was restricted to the cell surface (Fig. 3C and D) and both were taken up by the cells at 37 °C (Fig. 3A and B). However, the localization of naked and complexed pDNA at 37 °C was different. Naked pDNA was internalized in the cytosolic compartment (Fig. 3A), while complexed pDNA was localized in the vicinity of the cell membrane (Fig. 3B). This more pronounced temperature-dependence in naked pDNA uptake by U937 cells was consistent with the results obtained with [^{32}P]pDNA (Fig. 1).

3.2. Effect of polyanions on pDNA binding to human macrophage-like cells

We have already demonstrated that in mouse macrophages and DCs, pDNA was recognized by specific mechanisms for some polyanions. However, the recognition of pDNA in human macrophages is not yet fully understood.

The specificity of the binding of naked [^{32}P]pDNA was examined by cross-competition experiments using various macromolecules. As shown in Fig. 4, the cellular association of naked [^{32}P]pDNA was significantly inhibited by the presence of poly[I] and dextran sulfate, but not by poly[C] and dextran at 4 °C. These results suggest that a specific mechanism may be also involved in the recognition of pDNA by human macrophage-like cells.

3.3. Immune activation stimulated by naked DNA in human macrophage-like cells

In human immune cells, it is known that pDCs and B cells, but not monocytes or macrophages, express TLR9 and mainly recognize CpG motifs in DNA,

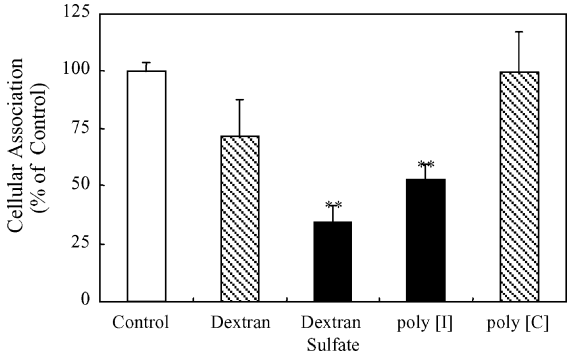

Fig. 4. Inhibition of cellular association of [^{32}P]pDNA with U937 Cells at 4 °C. The cells were incubated with [^{32}P]pDNA (0.1 μg/mL) for 8 h in the presence or absence various polyanions. Each point represents the mean ± S.D. ($n = 3$). $^{**}p < 0.01$ vs. control.

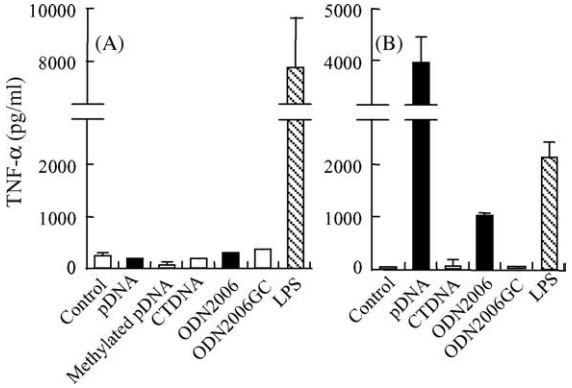

Fig. 5. Cellular activation by pDNA and other DNAs in U937 cells (A) and RAW264.7 cells (B). Cells were incubated with pDNA (100 μg/mL), methylated pDNA (100 μg/mL), CTDNA (100 μg/mL), ODN2006 (10 μM) or ODN2006GC (10 μM) at 37 °C. After 24 h (A) or 8 h (B), the supernatants were collected. The amount of TNF-α release was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

obtained using immunostimulatory CpG ODN. However, pDNA and ODN differ structurally. To examine whether human macrophage-like cells could be activated by pDNA, cells were stimulated with various DNA-containing unmethylated CpG motifs or a limited number of unmethylated CpG motifs. When PMA-treated U937 cells were cultured with ODN2006, a proven activator of human immune cells (Kerkmann et al., 2003), these cells could not be activated, nor could control ODN 2006GC. Naked pDNA even at a high concentration did not induce a significant amount of TNF-α in the cells nor did methylated pDNA or CTDNA containing few unmethylated CpG motifs (Fig. 5). In RAW264.7 cells, which are a mouse macrophage cell-line and express TLR9, the same concentration of ODN or pDNA was able to induce a significant amount of TNF-α in a CpG motif-dependent manner.

3.4. Immune activation by DNA/cationic liposome complex in human macrophage-like cells

We carried out the same experiments using pDNA complexed with cationic liposomes, LipofectAMINE2000 (LA), which are taken up by macrophages via a non-specific mechanism based on electrostatic interaction. The pDNA/LA complex stimulated the cells to produce TNF-α (Fig. 6B). The secreted amount of TNF-α slightly decreased at 24 h presumably due to degradation and cellular uptake. Since liposomes alone are unable to stimulate the macrophages sufficiently to release TNF-α, these results show that pDNA is indispensable for TNF-α production by the liposome formulation.

To explore whether the unmethylated CpG motif in pDNA complexed with LA is required for TNF-α induction from macrophages, we prepared methylated-CpG pDNA. The methylated-CpG pDNA/LA complex induced a similar amount of TNF-α compared with the

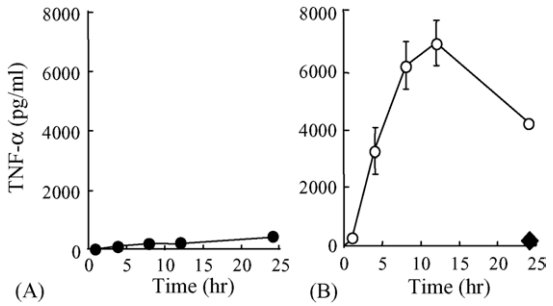

Fig. 6. Time-courses of cytokine release induced by naked pDNA (A) or pDNA/LA complex (B) from U937 cells. (A) Cells were incubated with naked pDNA (50 μg/well, closed circle) at 37 °C. (B) Cells were incubated with pDNA/LA complex (2.5 μg:5 μg/well, open circle) or LA only (5 μg/well, closed diamond) at 37 °C. After 2 h incubation, the cells were washed and incubated with growth medium. At the times indicated, the supernatants were collected. The amount of TNF-α release was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

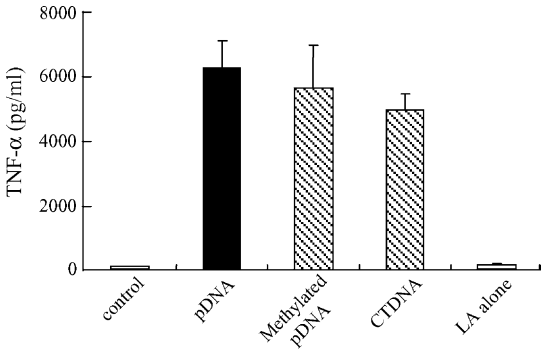

Fig. 7. TNF-α release by DNA/LA complex from U937 cells. DNA/LA complex was added to the cells. After 2 h incubation, the complex was removed and fresh growth medium was added to the cells. The supernatants were collected after 24 h incubation. The amount of TNF-α released from the cells was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

pDNA/LA complex. Vertebrate DNA, CTDNA, also induce a significant amount of TNF-α when complexed with cationic liposomes (Fig. 7). These results indicate that the DNA/cationic liposome complex is able to activate human macrophages in a CpG motif-independent manner.

4. Discussion

Although the important role of the immunostimulatory effects mediated by CpG motifs in gene therapy and DNA vaccination is well understood, most in vitro studies focusing on the mechanisms of activation mediated by CpG DNA have been carried out using CpG ODN and bacterial genomic DNA in mouse macrophages, DCs and human peripheral blood mononuclear cells (PBMCs). The present study shows that pDNA is recognized by human macrophage-like cells via a specific mechanism for polyanions and DNA complexed with cationic liposomes is able to activate human macrophage-like cells in a CpG motif-independent manner, while the cells are not activated by naked CpG DNA.

In some reports using CpG ODN, it has been shown that CpG ODN is internalized to human PBMCs by endocytosis and subsequent endosomal acidification is necessary for signal transduction via TLR9 similar to murine macrophages or DCs (Macfarlane and Manzel, 1998; Bauer et al., 1999).

There are differences between humans and mice as far as the active CpG motifs are concerned. CpG ODNs that activate PBMC from humans were initially classified into two types (Verthelyi et al., 2001). The B (also known as K) type ODNs have phosphorothioate backbones, encode multiple TCG motifs and primarily stimulate B cell and monocyte proliferation, and IgM, interleukin-10 (IL-10) and IL-6 secretion. By contrast, the A (also known as D) type ODN have mixed phosphodiester-phosphorothioate backbones and contain a single hexameric purine-pyrimidine-CG-purine-pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3′-end by a poly-G tail. However, pDNA and immunostimulatory CpG ODN differ structurally. CpG ODNs are single-stranded with phosphorothioate bonds, including a single CpG motif. On the other hand, pDNA are double-stranded with phosphodiester bonds with an abundance of nucleotides likely to generate multiple CpG motifs. Therefore, it is important to elucidate the cellular uptake characteristics of pDNA and the subsequent activation in human macrophages.

Naked pDNA was taken up by human macrophage-like cells, both PMA-treated U937 cells and THP-1 cells. The amounts of naked pDNA associated with the cells were comparable with those of the pDNA complexed with LA. However, the internalization of naked pDNA showed a more pronounced temperature-dependence than complexed pDNA and the recognition of pDNA by the cells involved specific mechanisms for polyanion, which have similar characteristics to those previously observed in mouse macrophages and DCs. Some other groups have also reported that human leucocytes are able to bind and internalize lambda phage DNA mediated by a specific protein on the cellular membrane (Bennett et al., 1985). This shows that naked pDNA is recognized by a specific membrane protein or receptor for polyanions.

In human immune cells, it is well known that only pDCs and B cells, but not monocytes or macrophages, express TLR9 (Hornung et al., 2002) and mainly recognize CpG motifs in DNA. In the present study, the immune response induced by naked pDNA or CpG ODN was investigated in monocytic cell-lines, U937 cells. PMA-treated U937 cells did not release a significant amount of TNF-α by stimulation of either DNA containing CpG motifs or CpG non-containing DNA probably due to lack of TLR9 expression. We

have also observed same phenomena in human primary macrophages differentiated from PBMCs (Fukuhara et al., unpublished results). Murine macrophages, which express TLR9 were activated by the same DNA in a CpG motif-dependent manner. Since human macrophage-like cells release TNF-α following stimulation with LPS, this showed that the cells were fully able to produce inflammatory cytokines. Moreover, LPS is recognized by TLR4, which shares many signaling molecules downstream of TLR9, thus, irresponsiveness to CpG DNA in human macrophages might be due to TLR9 itself. It has been reported that in DCs from TLR9 knock out mice, the responsiveness to pDNA disappeared (Spies et al., 2003). Therefore, these findings suggest that in human immune cells, pDNA also recognizes its own CpG motif by TLR9.

DNA/cationic liposome complex is also used as a gene vector or carrier in human clinical trials of non-viral gene therapy. However, pDNA/cationic liposome complex is well known to induce high levels of inflammatory cytokines in in vivo studies in mice (Alton et al., 1998; Freimark et al., 1998; Paillard, 1999; Yew et al., 2000; Sakurai et al., 2002). When pDNA was complexed with cationic liposomes, a significant amount of TNF-α was released from human macrophage-like cells, although TNF-α was not induced by naked pDNA. Since liposomes alone are unable to stimulate cells sufficiently to release TNF-α, these results show that pDNA is indispensable for TNF-α release by these liposome formulations. However, methylated pDNA or CTDNA, which contain few unmethylated CpG motifs are also able to induce. We have previously observed that in murine macrophages, cellular activation in complexed form was caused in a CpG-indepemdent manner. Similar results were obtained in human primary macrophages differentiated from PBMCs (Fukuhara et al., unpublished results). These results suggest that in human macrophage-like cells, cytokine induction by DNA/LA complex is independent of the CpG motif and TLR9. It is probably due to a change in the mechanism of cellular uptake or intracellular trafficking after uptake. pDNA/cationic liposome complex is able to induce inflammatory cytokines in a CpG motif-independent manner. It has been reported that in TLR9 knock out mice, some markers of toxicity are not affected or reduced but are not eliminated completely (Zhao et al., 2004). Since under in vivo conditions, other immune cells probably influence the responsiveness of macrophages to pDNA/cationic liposomes, more information is required about human immune cells purified from PBMCs.

In conclusion, it has been shown that pDNA/cationic liposome complex is recognized as a danger signal by human macrophage-like cells. These findings will be an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

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