Table 1
Immunological memory in surviving mice

	1st i.p.	2nd i.p. (on day 80)	i.v. (on day 144)
Saline	0/10		
CpG ODN-lipoplex	8/9	6/8	4/6

Long-term survivors of mice treated with CpG ODN-lipoplex in the survival experiment were rechallenged with colon26/Luc cells intraperitoneally on day 80 and intravenously on day 140 after the first inoculation of tumor cells.

died within 60 days after the second inoculation of the tumor cells, other mice survived more than an additional 60 days, suggesting that the tumor cells are rejected in these individuals. Then, the surviving mice were given injections of colon26/Luc cells into the tail vein, without the administration of CpG ODN-lipoplex. Again, 4 out of 6 mice were alive for more than an additional 60 days after the intravenous inoculation. At day 200 after the initial inoculation, all surviving mice were sacrificed. The lungs of mice surviving long-term had no visible tumor colonies on the lung surface (Fig. 6B) and no detectable luciferase activity. In a quite contrast, an injection of colon26/Luc cells into the tail vein of naïve mice resulted in the formation of a number of tumor colonies in the lung (Fig. 6C).

4. Discussion

Generally speaking, during the initial stages of peritoneal dissemination, tumor cells in the peritoneal cavity selectively infiltrate into the milky spots of the greater omentum, which is the primitive lymphoid tissue in the peritoneal cavity [22]. Tumor cells present in milky spots finally form an omental cake, so the proliferation of tumor cells in the greater omentum is a life-threatening event in peritoneal dissemination patients [21]. In this study, we found that PO-type CpG oligonucleotides complexed with cationic liposomes effectively inhibit the tumor growth in the greater omentum in the mouse model of peritoneal dissemination.

Previous studies demonstrated that oligonucleotide containing CpG motifs is a very strong activator of monocytes, macrophages, B cells, and DCs [23]. The CpG motif that appears to be optimal for stimulating murine immune cells is different from that for human cells. CpG dinucleotide flanked by two purine bases on the 5′-side and two pyrimidine bases on the 3′ side, such as GACGTT, efficiently activates the murine immune system, whereas the optimal motif for humans is GTCGTT. In addition, it has been reported that the biological activity of a given hexamer is strongly modulated by the remaining sequences within the ODN. For example, in mice, the immune stimulatory effects of CpG ODN were enhanced if the ODN had a TpC dinucleotide on the 5′ end and was pyrimidine-rich on the 3′ side. In addition, ODN with two or three CpG motifs in the sequence were found to be very potent compared to those with only one CpG motif [23]. The CpG motifs in ODN containing several motifs should not be back-to-back but preferably have at least two intervening bases, preferably Ts [3,9]. Moreover, single-stranded CpG ODN can stimulate immune functions more strongly than the double-stranded counterpart [24]. Therefore, in this study, based on these previous findings, we designed a single-stranded CpG ODN with the sequence 5′-TCGACGTTTTGACGTTTTGACGTTTT-3′ to induce effective immune responses, and found that the ODN can induce enough antitumor activity in mice when administered as a lipoplex. The importance of the sequence on the ODN-mediated antitumor activity was examined by using GpC ODN-lipoplex. Then we found that GpC ODN-lipoplex induced little TNF-α production from RAW264.7 cells. These results strongly suggest that TLR-9, the receptor recognizing the CpG motif, is involved in the antitumor activity of CpG ODN-lipoplex.

Oligonucleotide with the normal phosphodiester (PO) backbone is rapidly degraded by nucleases in serum and intracellular compartments. Phosphorothioate (PS) modification has been commonly used to stabilize oligonucleotides, but it has also been suggested that PS-type CpG ODN induces systemic toxicity, such as a transient anticoagulant effect, activation of complement cascade, and inhibition of basic fibroblast growth factor binding to surface receptors, because of non-specific protein binding [12,13]. In this study, instead of using PS-type CpG ODN, we used a PO-type CpG ODN complex with cationic liposomes to stabilize against degradation.

Cationic liposomes have been investigated for the stabilization of nucleic acids against degradation [15]. Indeed, cationic liposome-based gene delivery vectors are known for their ability to protect pDNA from serum nuclease degradation [25]. This feature allows PS modification to be avoided. There is another advantage of using CpG ODN and a cationic liposome complex. Lipoplex is recognized as a foreign material, and is phagocytosed by immune cells, especially by mononuclear phagocytes. These cells express TLR-9, and recognize CpG motifs in DNA via the receptors and produce Th-1 type cytokines, which are a form of cancer immunotherapy [15,26–28]. Indeed, in this study, mouse macrophage-like RAW264.7 cells stimulated with CpG ODN-lipoplex induced a large amount of TNF-α while naked CpG ODN resulted in only a minor degree of induction. Therefore, it is suggested that the formation of a cationic liposome complex can deliver CpG ODN more effectively to macrophages than naked CpG ODN. Moreover, intraperitoneal injection of CpG ODN-lipoplex can induce Th-1 type cytokines such as TNF-α and IL-12 (p70) in ascitic fluid. Therefore, CpG ODN is delivered to peritoneal immunocompetent cells, such as macrophages, where CpG motifs are recognized and these results in the production of several Th-1 type cytokines. The cytokine levels detected after administration of CpG ODN-lipoplex to mice were much lower than those detected after addition to RAW264.7 cells. This discrepancy could be explained by the distribution and elimination of cytokines in vivo, and by the higher reactivity of RAW264.7 cells to CpG DNA than peritoneal macrophages [29].

There are many phagocytic cells in the body that express TLR-9, for example, DCs, peritoneal macrophages, Kupffer cells, splenic macrophages, and Langerhans' cells. When injected intraperitoneally, intravenously, or intradermally, CpG ODN-lipoplex is thought to be mainly recognized by peritoneal macrophages, Kupffer cells and splenic macrophages, or Langerhans' cells, respectively. Previous studies demonstrated

that intravenous or intradermal injection of CpG DNA induces systemic immune responses [16,30]. In the present study, we also found that an intravenous administration of CpG ODN-lipoplex significantly increased the serum concentration of TNF-α (data not shown). However, no significant changes were observed in the cytokine levels in the peritoneal cavity after injection of the lipoplex into the tail vein or into the dorsal skin. Thus, it is suggested that the local immunostimulation produced by CpG ODN-lipoplex in the peritoneal cavity is important for the inhibition of the peritoneal dissemination of tumor cells. It appears that immunocompetent cells in the peritoneal cavity are efficiently activated by intraperitoneal injection of CpG ODN-lipoplex and then induce a localized immune response.

Intraperitoneal administration of CpG ODN-lipoplex prolonged the survival of the peritoneal dissemination mice. Surviving mice persisted in spite of second inoculation of tumor cells into the peritoneal cavity. Furthermore, no visible tumor colonies were detected in the lung of the surviving mice that received an intravenous injection of colon26/Luc cells, whereas all naïve mice produced a number of metastatic colonies in the lung by the injection. So it appears that CpG ODN-lipoplex induces systemic and long-lasting antitumor activity. Further studies are needed to clarify the long-lasting antitumor mechanisms of CpG ODN-lipoplex.

In conclusion, a single administration of PO-type CpG ODN/cationic liposome complex can induce Th-1 type cytokines in the peritoneal cavity, and inhibit peritoneal dissemination in mice. Local immune activation in the peritoneal cavity is found to be important for the inhibition of peritoneal dissemination, because intraperitoneal injection of CpG ODN-lipoplex was more effective in reducing peritoneal tumor cells than intravenous or intradermal injection of CpG ODN-lipoplex. Moreover, the immune responses induced by CpG ODN-lipoplex can be effective in preventing tumor growth in and outside the peritoneal cavity. Immunotherapy with CpG ODN-lipoplex is not limited by the type of tumor cells. Therefore, CpG ODN-lipoplex is a potentially useful tool for the treatment of a broad range of tumor types.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labour and Welfare, Japan.

References

[1] S. Gurunathan, D.M. Klinman, R.A. Seder, DNA vaccines: immunology, application, and optimization, Annu. Rev. Immunol. 18 (2000) 927–974.
[2] T. Tokunaga, H. Yamamoto, S. Shimada, H. Abe, T. Fukuda, Y. Fujisawa, Y. Furutani, O. Yano, T. Kataoka, T. Sudo, N. Makiguchi, T. Suganuma, Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG, J. Natl. Cancer Inst. 72 (1984) 955–962.
[3] A.M. Krieg, A.K. Yi, S. Matson, T.J. Waldschmidt, A. Bishop, R. Teasdale, G.A. Koretzky, D.M. Klinman, CpG motifs in bacterial DNA trigger direct B-cell activation, Nature 374 (1995) 546–549.
[4] H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA, Nature 408 (2000) 740–745.
[5] A.K. Yi, J.H. Chace, J.S. Cowdery, A.M. Krieg, IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides, J. Immunol. 156 (1996) 558–564.
[6] D.M. Klinman, A.K. Yi, S.L. Beaucage, J. Conover, A.M. Krieg, CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 2879–2883.
[7] Z.K. Ballas, W.L. Rasmussen, A.M. Krieg, Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA, J. Immunol. 157 (1996) 1840–1845.
[8] G.B. Lipford, M. Bauer, C. Blank, R. Reiter, H. Wagner, K. Heeg, CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants, Eur. J. Immunol. 27 (1997) 2340–2344.
[9] A.K. Yi, M. Chang, D.W. Peckham, A.M. Krieg, R.F. Ashman, CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry, J. Immunol. 160 (1998) 5898–5906.
[10] R. Rankin, R. Pontarollo, X. Ioannou, A.M. Krieg, R. Hecker, L.A. Babiuk, S. van Drunen Littel-van den Hurk, CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved, Antisense Nucleic Acid Drug Dev. 11 (2001) 333–340.
[11] Q. Zhao, J. Temsamani, R.Z. Zhou, S. Agrawal, Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice, Antisense Nucleic Acid Drug Dev. 7 (1997) 495–502.
[12] S.M. Fennewald, R.F. Rando, Inhibition of high affinity basic fibroblast growth factor binding by oligonucleotides, J. Biol. Chem. 270 (1995) 21718–21721.
[13] S.P. Henry, P.C. Giclas, J. Leeds, M. Pangburn, C. Auletta, A.A. Levin, D.J. Kornbrust, Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action, J. Pharmacol. Exp. Ther. 281 (1997) b810–b816.
[14] M. Rutz, J. Metzger, T. Gellert, P. Luppa, G.B. Lipford, H. Wagner, S. Bauer, Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner, Eur. J. Immunol. 34 (2004) 2541–2550.
[15] O. Zelphati, F.C. Szoka, Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11493–11498.
[16] M.M. Whitmore, S. Li, L. Falo Jr., L. Huang, Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses, Cancer Immunol. Immunother. 50 (2001) 503–514.
[17] K. Hyoudou, M. Nishikawa, Y. Umeyama, Y. Kobayashi, F. Yamashita, M. Hashida, Inhibition of metastatic tumor growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells, Clin. Cancer Res. 10 (2004) 7685–7691.
[18] G. Poste, J. Doll, I.R. Hart, I.J. Fidler, In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties, Cancer Res. 40 (1980) 1636–1644.
[19] T.H. Corbett, D.P. Griswold Jr., B.J. Roberts, J.C. Peckham, F.M. Schabel Jr., Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure, Cancer Res. 35 (1975) 2434–2439.
[20] S. Fumoto, F. Nakadori, S. Kawakami, M. Nishikawa, F. Yamashita, M. Hashida, Analysis of hepatic disposition of galactosylated cationic liposome/plasmid DNA complexes in perfused rat liver, Pharm. Res. 20 (2003) 1452–1459.
[21] L.F. Krist, M. Kerremans, D.M. Broekhuis-Fluitsma, I.L. Eestermans, S. Meyer, R.H. Beelen, Milky spots in the greater omentum are predominant sites of local tumour cell proliferation and accumulation in the peritoneal cavity, Cancer Immunol. Immunother. 47 (1998) 205–212.
[22] H. Tsujimoto, T. Takhashi, A. Hagiwara, M. Shimotsuma, C. Sakakura, K. Osaki, S. Sasaki, M. Shirasu, T. Sakakibara, T. Ohyama, A. Sakuyama, M. Ohgaki, T. Imanishi, J. Yamasaki, Site-specific implantation in the milky spots of malignant cells in peritoneal dissemination: immunohistochemical observation in mice inoculated intraperitoneally with bromodeoxyuridine-labelled cells, Br. J. Cancer 71 (1995) 468–472.

[23] A.M. Krieg, CpG motifs in bacterial DNA and their immune effects, Annu. Rev. Immunol. 20 (2002) 709–760.
[24] S. Zelenay, F. Elias, J. Flo, Immunostimulatory effects of plasmid DNA and synthetic oligodeoxynucleotides, Eur. J. Immunol. 33 (2003) 1382–1392.
[25] P.L. Felgner, G.M. Ringold, Cationic liposome-mediated transfection, Nature 337 (1989) 387.
[26] G. Trinchieri, Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes, Blood 84 (1994) 4008–4027.
[27] M.T. Lotze, L. Zitvogel, R. Campbell, P.D. Robbins, E. Elder, C. Haluszczak, D. Martin, T.L. Whiteside, W.J. Storkus, H. Tahara, Cytokine gene therapy of cancer using interleukin-12: murine and clinical trials, Ann. N.Y. Acad. Sci. 795 (1996) 440–454.
[28] K. Yasuda, Y. Ogawa, M. Kishimoto, T. Takagi, M. Hashida, Y. Takakura, Plasmid DNA activates murine macrophages to induce inflammatory cytokines in a CpG motif-independent manner by complex formation with cationic liposomes, Biochem. Biophys. Res. Commun. 293 (2002) 344–348.
[29] K. Yasuda, H. Kawano, I. Yamane, Y. Ogawa, T. Yoshinaga, M. Nishikawa, Y. Takakura, Restricted cytokine production from mouse peritoneal macrophages in culture in spite of extensive uptake of plasmid DNA, Immunology 111 (2004) 282–290.
[30] L. Liu, X. Zhou, H. Liu, L. Xiang, Z. Yuan, CpG motif acts as a 'danger signal' and provides a T helper type 1-biased microenvironment for DNA vaccination, Immunology 115 (2005) 223–230.

Plasmid DNA Uptake and Subsequent Cellular Activation Characteristics in Human Monocyte-Derived Cells in Primary Culture

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Received 30 May 2006; revised 21 August 2006; accepted 22 September 2006

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20816

ABSTRACT: Plasmid DNA (pDNA) uptake and subsequent cellular activation characteristics were studied in three types of human monocyte-derived cells, that is, human monocytes, macrophages, and dendritic cells (DCs) in primary culture. Naked pDNA was bound to and taken up by the macrophages and DCs while only significant binding occurred in the monocytes. pDNA binding to these monocyte-derived cells was significantly inhibited by polyinosinic acid (poly[I]), dextran sulfate, maleylated bovine serum albumin (Mal-BSA) and to a lesser extent by polycytidylic acid (poly[C]), but not by dextran or galactosylated BSA (Gal-BSA), mannosylated BSA (Man-BSA), suggesting that a specific mechanism for polyanions is involved in the pDNA binding. In cellular activation studies, naked pDNA could not induce TNF-α production from any monocyte-derived cells, regardless of the abundant presence of CpG motifs in the pDNA. However, when complexed with cationic liposomes, pDNA produced a significant amount of TNF-α from the human macrophages. TNF-α induction was not observed in the monocytes or DCs. Moreover, calf thymus DNA (CT DNA) complexed with cationic liposomes also induced TNF-α production to a similar extent in the human macrophages. These results indicate that, among human monocyte-derived cells, macrophages are activated by DNA when complexed with cationic liposomes in a CpG motif-independent manner.

Keywords: gene therapy; plasmid DNA; cellular uptake; degradation; monocyte-derived cells

INTRODUCTION

Plasmid DNA (pDNA) is an important macromolecular agent in gene therapy as well as DNA vaccination.[1–3] In spite of the lower transfection efficiency compared with viral vectors, pDNA has advantages in terms of its safety and productivity. Because pDNA is a bacterial DNA that is generally replete with unmethylated CpG dinucleotides (CpG motifs) in contrast to vertebrate DNA, it can activate mammalian immune cells through recognition by TLR9. Upon activation, the cells release inflammatory cytokines, which may influence the efficacy of pDNA-based therapy. To achieve effective and safe pDNA-based therapy, it is essential to understand the mechanism by which pDNA is taken up by macrophages and dendritic cells (DCs), the most important cells responsible for the uptake of pDNA *in vivo* and subsequent cytokine production.

Abbreviations: pDNA, plasmid DNA; CT DNA, calf thymus DNA; DC, dendritic cell; TLR, toll-like receptor; poly[I], polyinosinic acid; poly[C], polycytidylic acid.

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Journal of Pharmaceutical Sciences

Previously, we have demonstrated that pDNA is rapidly removed from the circulation and taken up by the liver, preferentially by the liver nonparenchymal cells, after intravenous administration to mice.[4] Recently, we confirmed that Kupffer cells, liver resident macrophages, and liver sinusoidal endothelial cells have a significant role in the hepatic uptake and degradation of pDNA.[5] *In vitro* studies using cultured mouse peritoneal macrophages demonstrated that pDNA uptake was dramatically inhibited by defined poylanions, such as polyinosinic acid (poly[I]) and dextran sulfate, but not by polycytidylic acid (poly[C]).[6] A similar inhibition profile was observed for the *in vivo* hepatic uptake of pDNA following intravenous administration.[4] These results suggested that a specific receptor, like the class A scavenger receptor (SRA), could be involved in the endocytotic uptake of pDNA by macrophages. However, the pDNA uptake was not significantly altered in macrophages isolated from SRA-knockout mice and in SRA-knockout mice *in vivo*.[7] More recently, it was found that a murine DC cell-line, DC2.4, takes up pDNA by a similar specific mechanism more efficiently and rapidly than macrophages.[8]

Macrophages and DCs are known to secrete a large amount of inflammatory cytokines following recognition of unmethylated CpG motifs in pDNA via TLR9 as a danger signal.[9] These inflammatory cytokines significantly reduce transgene expression of pDNA, because they may lead the transfected cells to undergo cell death or shut off the viral promoter-based expression.[10,11] Meanwhile, these cytokines are essential for increasing the pDNA-induced immune response following DNA vaccination.[3,12] Our previous study has demonstrated that, in contrast to macrophage cell-lines, primary cultured mouse peritoneal macrophages secreted almost no inflammatory cytokines upon stimulation with pDNA, in spite of extensive uptake of the CpG DNA.[13] However, not only pDNA but also vertebrate DNA, and calf thymus DNA (CT DNA) can activate the murine macrophages to induce inflammatory cytokines, when complexed with cationic liposomes.[14] Similar results were obtained in murine bone marrow DCs cultured with Flt-3 ligand,[15] indicating that endosomal translocation of vertebrate DNA can activate macrophages and DCs in a CpG-independent manner. Furthermore, recent studies using macrophages and DCs isolated from TLR9-knockout mice have demonstrated that both pDNA and CT DNA can activate the TLR9-independent pathway.[15–17]

In spite of a growing body of information on pDNA uptake and cellular activation characteristics in mouse cells, there is insufficient information about human cells. Very recently, we studied the cellular uptake and activation characteristics of naked pDNA and its cationic liposome complex in human macrophagelike cell-lines, U937 cells and THP-1 cells.[18] The present study was carried out to expand the knowledge to human cells in primary culture, which would provide us with more important information for DNA-based therapy. In the present study, we used three types of human monocyte-derived cells, that is, monocytes, macrophages, and DCs, to examine the uptake and subsequent cellular activation by pDNA in both naked and complexed forms.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) and Ficoll-Paque were obtained from Amersham (Buckinghamshire, England). poly[I] (MW 103.3 kDa), poly[c] (MW 99.5 kDa), and dextran (MW 70 kDa) were purchased from Pharmacia (Uppsala, Sweden). Dextran sulfate (MW 150 kDa) was purchased from Nacalai Tesque (Kyoto, Japan). pCMV-Luc encoding firefly luciferase gene constructed previously was used as a model pDNA. CT DNA, used as DNA with less CpG motifs, was purchased from Sigma (St Louis, MO). Synthetic phosphorothioate ODN were purchased from Hokkaido System Science Co. Ltd (Sapporo, Japan). The sequence of CpG S-ODN2006 is 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′, a proven activator of human immune cells.[19] Phosphorothioate non-CpG ODN 2006GC (5′-TGCTGCTTTTGTGCTTTTGTGCTT-3′) was used as a control. LipofectAMINE 2000 (LA) and Opti-MEM were purchased from Lifetechnologies (Rockville, MD). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). GM-CSF and IL-4 were purchased from PeproTech. Bovine serum albumin (BSA) derivatives, that is, galactosylated BSA (Gal-BSA), mannosylated BSA (Man-BSA), and maleylated bovine serum albumin (Mal-BSA) were synthesized as described previously.[4] All other chemicals used were of the highest purity available.

Harvesting and Culture of Primary Human Monocyte-Derived Cells

After obtaining informed consent, fresh whole blood was withdrawn from healthy donors and transferred into tubes. Peripheral blood mononuclear cells (PBMC) were obtained from the buffy coat of healthy donors through Ficoll-Hypaque centrifugation in order to prepare monocytes, macrophages, [20] and DCs.[21] Briefly, $CD14^+$ monocytes were isolated from PBMC by positive selection using a MACS system, according to the manufacturer's protocol. These cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (1.2 μg/mL). Monocytes were cultured in the presence of GM-CSF (50 ng/mL) to induce monocyte-derived macrophages. For the preparation of monocyte-derived immature DCs, GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) were added to the culture medium. After 6 days, the cells were recovered by washing with PBS to remove all nonadherent DCs and adherent macrophages before starting the experiments. These cells were seeded at 5×10^5 cells in 24-well plates for the experiments.

Plasmid DNA

For the cellular association experiment, pCMV-Luc was radiolabeled using [α-^{32}P]dCTP by nick translation.[22] For the confocal microscopic study, pCMV-Luc was labeled using a Fasttag FL labeling kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). DNA/LA complexes were prepared in a ratio of DNA:LA 1:2 according to the manufacturer's instructions.

Purification of DNA

To minimize the activation by contaminated LPS, each DNA sample was extensively purified by washing with a nonionic detergent, Triton X-114, according to the previously published methods[23,24] with slight modifications as previously described.[12] 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; that is, 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.

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 [^{32}P]pDNA/LA complex was added. After incubation at 37°C 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 containing 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 protein content was measured using a modified Lowry method with BSA as a standard. Unlabeled macromolecules, such as poly[I], poly[C], dextran, and dextran sulfate, were added to the incubation wells concomitantly with [^{32}P]pDNA to examine the effects of those macromolecules on the binding of [^{32}P]pDNA.

Precipitation of Degraded pDNA

After the cells were incubated with [^{32}P]pDNA in the naked form in the cellular association experiments at 37°C, the medium and the cell lysate were separately collected and subjected to trichloroacetic acid (TCA) precipitation experiments to assess the degradation of [^{32}P]pDNA following cellular uptake by monocyte-derived cells as previously described.[8] A portion of the supernatant was used directly for radioactivity counting as described above. After extraction with 10 mM Tris-HCl, 1 mM EDTA (TE)-saturated phenol buffer (pH 7.8), aliquots of the supernatant were mixed with TCA to give a final concentration of 5% (w/v), kept on ice for 10 min, and then

centrifuged at 9000*g* for 30 min at 4°C. The supernatant (TCA-soluble fraction) was used for radioactivity counting and the amount of degradation products of ^{32}P-labeled pDNA was calculated. The TCA-soluble degradation products are small DNA fragments (short oligonucleotides) since 50% precipitation occurs with the 16-mer oligonucleotides in the case of TCA.

Cytokine Secretion

Prior to the addition of naked or complexed DNA, monocyte-derived cells were washed with PBS. Naked DNA and DNA/LA complex were diluted in Opti-MEM. Following the addition of DNA/LA complexes to cells, the cells were incubated for 2 h and then the solution was removed and the cells were incubated with RPMI 1640 with 10% FBS medium continuously for specified periods up to 24 h. When naked pDNA was added to cells, the cells were incubated with Opti-MEM up to 24 h. After incubation, the medium was collected to measure the cytokines. We measured TNF-α, one of the typical inflammatory cytokines, which was reported to reduce transgene expression significantly,[10,19] as an indicator of cellular activation, although type I interferon response is also indicative to the activation in human cells.[15,19] The levels of TNF-α in the conditioned medium were measured using a Human TNF-α ELISA set (eBioscience, San Diego, CA) according to the manufacturer's instructions.

RESULTS

Cellular Uptake and Degradation of Naked pDNA in Human Primary Monocyte-Derived Cells

Figure 1 shows the time-courses of the cellular association of pDNA in three types of human monocyte-derived cells. A extensive cellular association of [^{32}P]pDNA was observed for monocytes (Fig. 1A), however, there was no significant difference between the amounts at 37°C and 4°C, suggesting that only binding to the cell surface occurred. On the other hand, macrophages and DCs appeared to significantly take up [^{32}P]pDNA in a temperature-dependent manner (Fig. 1B and C). TCA-soluble products increased with time in the culture medium of macrophages and DCs incubated with naked [^{32}P]pDNA in the cellular association experiments at 37°C (Fig. 2B and C) whereas this was not the case in the culture medium of monocytes (Fig. 2A). DCs degraded [^{32}P]pDNA more efficiently than macrophages. These results indicate that cellular association profiles are different between human monocyte-derived cells and macrophages and that DCs, but not monocytes, significantly take up and degrade pDNA.

Cellular Association of pDNA/LA Complex in Human Monocyte-Derived Cells

Figure 3 shows the time-courses of the cellular association of [^{32}P]pDNA/liposome complex in the

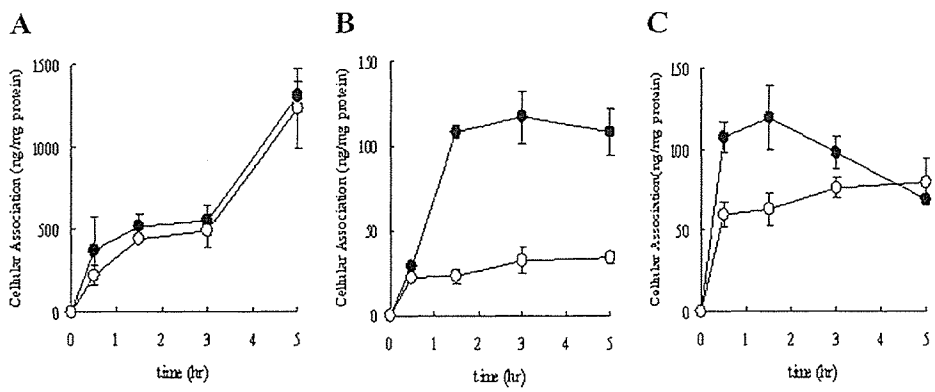

Figure 1. Time-courses of the cellular association of [^{32}P]pDNA with (A) monocytes, (B) macrophages, and (C) DCs. The cells were incubated with [^{32}P]pDNA (0.1 μg/mL) at 37°C (closed circle) or 4°C (open circle). Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small. The percentages of the cellular association with monocytes, macrophages, and DCs were 27.1%, 5.0%, and 9.5% of added pDNA at 5 h, respectively.

DOI 10.1002/jps

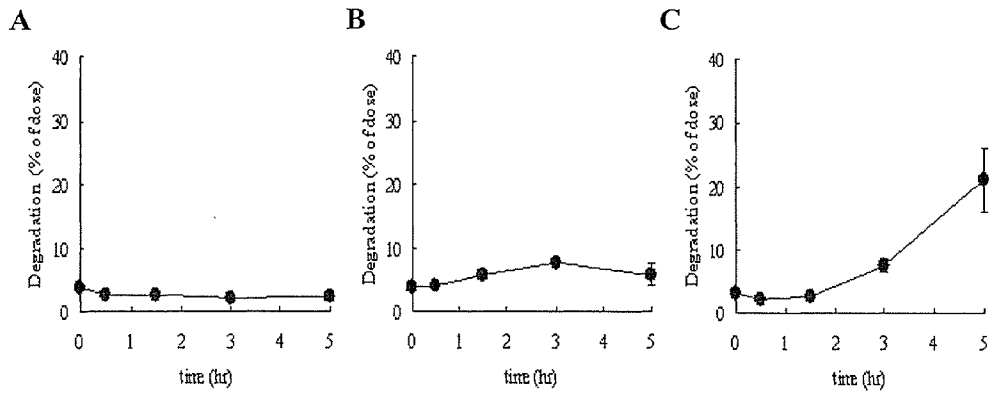

Figure 2. Time-courses of TCA soluble radioactivity in (A) monocytes, (B) macrophages, and (C) DCs in culture medium. The cells were incubated with [^{32}P]pDNA (0.1 μg/mL) at 37°C. Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small.

human monocyte-derived cells. In contrast to naked pDNA, similar cellular association profiles were observed. [^{32}P]pDNA/liposome complex was taken up by all types of cells in a temperature-dependent manner. The apparent uptake rate for the initial period was higher in DCs (Fig. 3C) and lower in macrophages (Fig. 3B), compared with that in monocytes (Fig. 3A).

Effect of Various Polyanions on the Binding of Naked pDNA to Human Primary Monocyte-Derived Cells

The binding characteristics of naked [^{32}P]pDNA were examined by competition experiments using various macromolecules. As shown in Figure 4, the binding of [^{32}P]pDNA was significantly inhibited by poly[I], dextran sulfate, or poly[C], but not by dextran in all types of monocyte-derived cells at 4°C. Figure 5 shows the effects of various modified BSAs on the cellular binding of [^{32}P]pDNA to the three types of cells. The binding was significantly inhibited by Mal-BSA with negative charges while synthetic glycoproteins, such as Man-BSA and Gal-BSA, did not show any inhibition. These results also suggest that the recognition of pDNA by these monocyte-derived cells is based on the negative charges of the molecule.

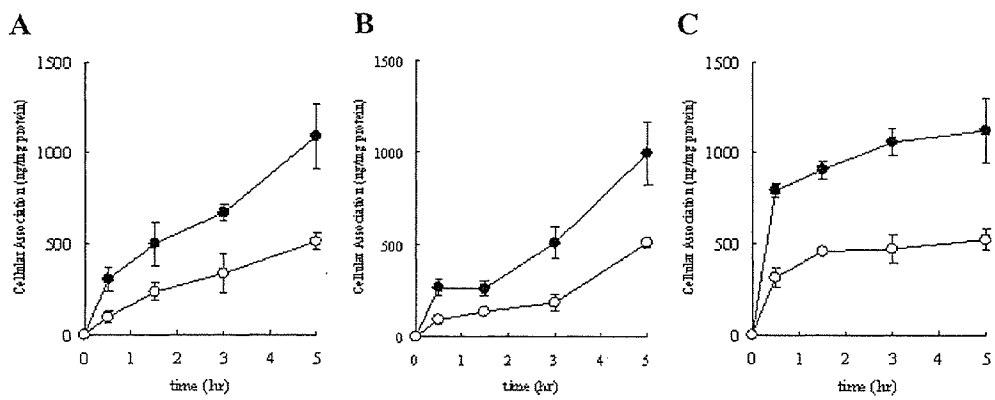

Figure 3. Time-courses of cellular association of [^{32}P]pDNA/LA complex with (A) monocytes, (B) macrophages, and (C) DCs. The cells were incubated with [^{32}P]pDNA/LA (0.1 μg/mL: 0.2 μg/mL) at 37°C (closed circle) or 4°C (open circle). Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small. The percentages of the cellular association with monocytes, macrophages, and DCs were 34.7%, 34.4%, and 72.0% of added pDNA at 5 h, respectively.

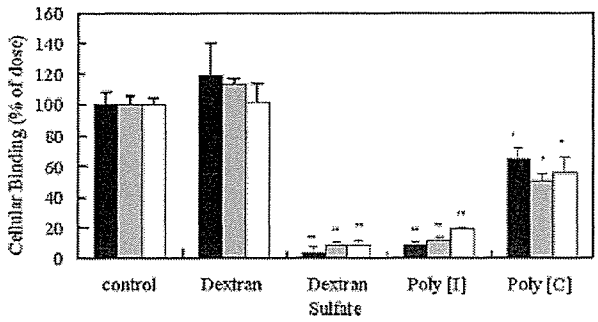

Figure 4. Inhibition of cellular binding of [^{32}P]pDNA with monocytes (black bar), macrophages (gray bar), and DCs (white bar) by macromolecules at 4°C. The cells were incubated with [^{32}P] pDNA (0.1 μg/mL) for 3 h in the presence or absence of the various macromolecules (50 μg/mL). The results are expressed as mean ± SD ($n = 3$). Statistical significance was analyzed by the Student t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

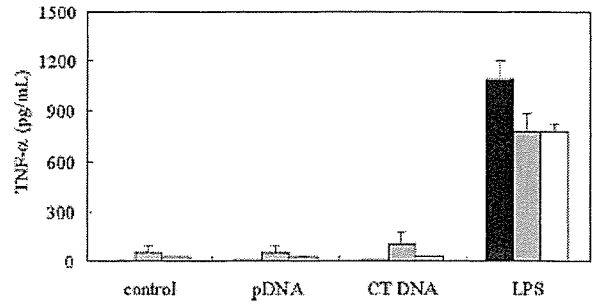

Figure 6. TNF-α release induced by naked DNA and from monocytes (black bar), macrophages (gray bar), and DCs (white bar). The cells were incubated with pDNA or CT DNA (10 μg/mL) or LPS (10 ng/mL a positive control). The control cells were treated with medium alone. The supernatants were collected after 24 h. TNF-α levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

Cellular Activation Stimulated by Naked DNA or DNA/LA Complex in Human Monocyte-Derived Cells

To evaluate the activation of human monocyte-derived cells by pDNA, the cells were incubated with various naked pDNAs and CT DNAs or their cationic liposome complexes. Figure 6 illustrates TNF-α production following stimulation with naked pDNA, CT DNA, and LPS as a positive control. Neither pDNA nor CT DNA induces a significant amount of TNF-α in any of the cells even at a high concentration of 100 μg/mL. A synthetic CpG S-ODN2006 (10 μM) having active sequence for human cells [19] also failed to induce TNF-α production from the human monocyte-derived cells (data not shown), suggesting these cells express no significant TLR9. Only LPS showed cellular activation to secrete a significant amount of TNF-α. We also carried out the experiments using pDNA or CT DNA complexed with LA. The pDNA/LA complex stimulated macrophages to produce a large amount of TNF-α whereas no significant TNF-α production was observed for monocytes and DCs (Fig. 7). Cationic liposomes alone were unable to stimulate the

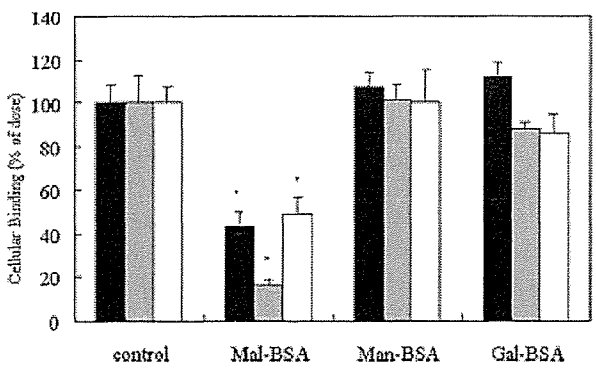

Figure 5. Inhibition of cellular binding of [^{32}P]pDNA to monocytes (black bar), macrophages (gray bar), and DCs (white bar) by modified BSA at 4°C. The cells were incubated with [^{32}P] pDNA (0.1 μg/mL) for 3 h in the presence or absence of the various forms of modified BSA (250 μg/mL). The results are expressed as mean ± SD ($n = 3$). Statistical significance was analyzed by the Student t-test. $^{*}p < 0.01$ versus control.

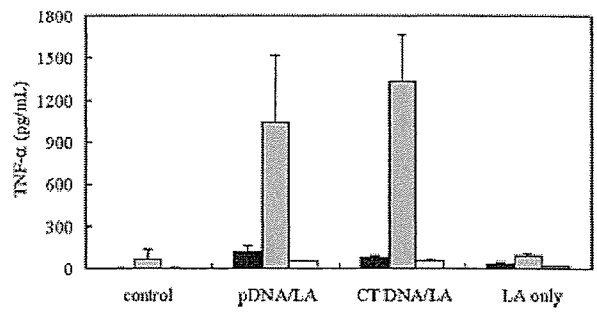

Figure 7. TNF-α release induced by DNA/LA complex from monocytes (black bar), macrophages (gray bar) and DCs (white bar). The cells were incubated with pDNA or CT DNA/LA complex (2.5 μg: 5 μg/well) or LA only (5 μg/well). After 2 h incubation, liposomes were removed and Opti-MEM was added to the cells. The control cells were treated with medium alone. The supernatants were collected 24 h after incubation with liposomes. TNF-α levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

DOI 10.1002/jps

macrophages sufficiently to release TNF-α, suggesting that pDNA is indispensable for TNF-α production by the liposome formulation. Interestingly, CT DNA complexed with cationic liposomes also induced TNF-α secretion from the macrophages. These results suggest that the DNA/cationic liposome complex activates human macrophages independent of the amount of CpG motifs contained in DNA.

DISCUSSION

The present study demonstrated that the naked pDNA uptake characteristics were different among human monocyte-derived cells in primary culture (Figs. 1 and 2). An efficient uptake and subsequent degradation of naked pDNA was observed in DCs. Macrophages also showed a similar profile with less degradation activity than DCs, while only significant binding occurred in monocytes. pDNA was more efficiently taken up and rapidly degraded by DCs than by macrophages. In mouse macrophages and DCs, endosomal acidification inhibitors, such as bafilomycin A and chloroquine, significantly inhibited degradation of naked [^{32}P]pDNA (unpublished results), suggesting that the degradation would occur in acidic milieu in the cells, probably in lysosomes after endocytosis.[25] A similar phenomenon might take place in the case of human macrophages and DCs.

In spite of the different characteristics involving pDNA uptake, the effect of polyanions on naked pDNA binding to the cellular surface was similar in all types of monocyte-derived cells (Figs. 4 and 5). pDNA binding to these monocyte-derived cells was significantly inhibited by an excess of some polyanions, such as poly[I] or dextran sulfate, but not by dextran (Fig. 4). poly[C] showed a significant but much lower degree of inhibition. Among BSA derivatives, only Mal-BSA with strong negative charges showed an inhibitory effect (Fig. 5). This inhibition profile by polyanions was almost identical to our previous observations in mouse macrophages,[7] DCs,[9] and a human macrophagelike cell-line.[18] poly[G], poly[I], and other particular polynucleotides are known to form a base-quartet-stabilized four-strand helix (quadruplex),[26] which would give a high density of negative charges. These negative charges might be important for pDNA binding in all types of monocyte-derived cells. These results suggest that a mechanism, by which the polyanionic nature of pDNA is recognized by the putative receptor, might be involved in the uptake of pDNA by the monocyte-derived macrophages and DCs in the primary culture. However, pDNA binding of a similar nature in terms of inhibition by polyanions did not result in uptake by the monocytes. One possible explanation is involvement of fibronection in DNA binding. Fibronection is a ubiquitous protein comprising extracellular matrix, which is reported to bind DNA with a similar affinity to RNA (poly [I] vs. poly [C]) observed in this study (Fig. 4).[27] We consider both putative receptor(s) and fibronection would be involved in DNA uptake in three types of monocyte-derived cells. We speculate that contribution of binding to fibronection, which does not result in internalization, might be higher in monocytes than DCs and macrophages. Further studies are required to clarify the uptake characteristics of naked pDNA.

In contrast to naked pDNA, the cellular association profiles of pDNA/LA complex were similar in all types of monocyte-derived cells (Fig. 3). These results suggest that the pDNA/cationic lipid complex is taken up by a nonspecific mechanism based on electrostatic interaction.

pDNA is a bacterial DNA that can be distinguished from the vertebrate form as far as replete unmethylated CpG dinucleotides (CpG motifs) are concerned. Mouse macrophages, DCs, and B cells are known to recognize unmethylated CpG motifs in pDNA or bacterial DNA via TLR9 as a danger signal to release inflammatory cytokines.[9] With regard to human cells, extensive studies have been carried using synthetic oligonucleotides (ODN) containing a variety of CpG motifs. However, the effects of CpG motifs contained in pDNA on human cells are not fully understood.

In the present study, the cellular activation induced by naked DNA or DNA/cationic liposome complex in the human monocyte-derived cells was investigated. None of the types of monocyte-derived cells released a significant amount of TNF-α by stimulation of naked DNA with replete CpG motifs or DNA with significantly fewer CpG motifs. In human immune cells, it is known that plasmacytoid DCs (pDCs) and B cells, but not monocytes or monocyte-derived immature DCs, express TLR9 and mainly recognize CpG motifs in DNA.[28,29] Siren et al. [30] reported that TLR9 mRNA was not detected in human monocyte-derived macrophages while it was reported that the response to pDNA disappeared in DCs from TLR9 knockout mice.[31] Probably, the lack of response to CpG DNA in human monocyte-derived

cells in the present study (Fig. 6) is due to the absence or very limited expression of TLR9. The results of synthetic CpG ODN also support this speculation.

DNA/cationic liposome complex is also used as a gene vector or carrier in human clinical trials of nonviral gene therapy. However, the pDNA/cationic liposome complex is well known to induce a high amount of inflammatory cytokines in *in vivo* studies in mice.[32–34] In the present study, when pDNA was complexed with cationic liposomes, a significant amount of TNF-α was released from human macrophage, although TNF-α was not induced by naked pDNA. Moreover, CT DNA containing less CpG motifs could also induce cellular activation in the complexed form. Recently, macrophages and DCs obtained from $TLR9^{-/-}$ mice were activated by DNA/cationic liposomes in a CpG motif-independent manner,[15–17] indicating that cellular activation can be induced through a TLR9-independent pathway. Our recent study using U937 cells also demonstrated that pDNA complexed with cationic liposomes activates the human macrophagelike cells to produce TNF-α independent of the amount of CpG motifs.[18] Together with this finding, the results obtained in the present study suggest that, among monocyte-derived cells, human macrophages are activated by DNA/cationic liposome complex independent of the amount of CpG motifs. This cellular activation might take place through a TLR9-independent pathway based on the literature information on TLR9 expression [30] and inability of CpG ODN in our cellular activation experiments, although we did not confirm the expression of TLR9 in this study.

In conclusion, we have demonstrated that the mechanisms involved in the uptake of pDNA by these cells has similar characteristics in terms of the specificity of ligand recognition among monocyte-derived cells, although the uptake properties of [^{32}P]pDNA were different. The pDNA/cationic liposome complex was recognized as a danger signal by human macrophages, although the underlying mechanism remains to be elucidated. These findings represent an important basis for the development of safer DNA-based therapy including gene therapy and DNA vaccination.

REFERENCES

1. Nishikawa M, Leaf H. 2001. Nonviral vectors in the new millennium: Delivery barriers in gene transfer. Hum Gene Ther 12:861–870.
2. Leitner WW, Ying H, Restifo NP. 1999. DNA and RNA-based vaccines: Principles, progress and prospects. Vaccine 18:765–777.
3. Donnelly JJ, Wahren B, Liu MA. 2005. DNA vaccines: Progress and challenges. J Immunol 175: 633–639.
4. Kobayashi N, Kuramoto T, Yamaoka K, Hashida M, Takakura Y. 2001. Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. J Pharmacol Exp Ther 297:853–860.
5. Hisazumi J, Kobayashi N, Nishikawa M, Takakura Y. 2004. Significant role of liver sinusoidal endothelial cells in hepatic uptake and degradation of naked plasmid DNA after intravenous injection. Pharm Res 21:1223–1228.
6. Takagi T, Hashiguchi M, Mahato RI, Tokuda H, Takakura Y, Hashida M. 1998. Involvement of specific mechanism in plasmid DNA uptake by mouse peritoneal macrophages. Biochem Biophys Res Commun 245:729–733.
7. Takakura Y, Takagi T, Hashiguchi M, Nishikawa W, Yamashita F, Doi T, Imanishi T, Suzuki H, Kodama T, Hashida M. 1999. Characterization of plasmid DNA binding and uptake by peritoneal macrophaeges from class A scavenger receptor knockout mice. Pharm Res 16:503–508.
8. Yoshinaga T, Yasuda K, Ogawa Y, Takakura Y. 2002. Efficient uptake and rapid degradation of plasmid DNA by murine dendritic cells via specific mechanism. Biochem Biophys Res Commun 299: 389–394.
9. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374:546–549.
10. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. 1997. Promotor attenuation in gene therapy: Interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum Gene Ther 8:2019–2029.
11. Ghazizadah S, Carroll JM, Taichman LB. 1997. Repression of retrovirus mediated transgene expression by interferons: Implication for gene therapy. J Virol 71:9163–9169.
12. Krieg AM, Kline JN. 2000. Immune effects and therapeutic applications of CpG motif in bacterial DNA. Immunopharmacology 48:303–305.
13. Yasuda K, Kawano H, Yaname I, Ogawa Y, Yoshinaga T, Nishikawa M, Takakura Y. 2004. Restricted cytokine production from mouse peritoneal macrophages in culture in spite of extensive uptake of plasmid DNA. Immunology 111:282–290.
14. Yasuda K, Ogawa Y, Kishimoto M, Takagi T, Hashida M, Takakura Y. 2002. Plasmid DNA activated murine macrophages to induce imflammatory cytokines in a CpG motif-independent

manner by complex formation with cationic liposomes. Biochem Biophys Res Commun 293:344–348.
15. Yasuda K, Yu P, Kirsching CJ, Schlatter B, Schmitz F, Heit A, Bauser S, Hochrein H, Wagner H. 2005. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J Immunol 174:6129–6136.
16. Yasuda K, Ogawa Y, Yamane I, Nishikawa M, Takakura Y. 2005. Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways. J Leukoc Biol 77:71–79.
17. Martin DA, Elkon EB. 2006. Intracellular mammalian DNA stimulates myeloid dendritic cells to produce type I interferons predominantly through a toll-like receptor 9-independent pathway. Arthritis Rheum 54:951–962.
18. Yamane I, Nishikawa M, Takakura Y. 2005. Cellular uptake and activation characteristics of naked plasmid DNA and its cationic liposome complex in human macrophages. Int J Pharm 305:145–153.
19. Kerkmann M, Rothenfusser S, Hornung V, et al. 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J Immunol 170:4465–4474.
20. Tabata S, Kadowaki N, Kitawaki T, Shimaoka T, Yonehara S, Yoshie O, Uchiyama T. 2005. Distribution and kinetics of SR-PSOX/CXCL16 and CXCR6 expression on human dendritic cell subsets and CD4+ T cells. J Leukoc Biol 77:777–786.
21. Steinbach F, Krause B, Blass S, Burmester GR, Hiepe F. 1998. Development of accessory phenotype and function during the differentiation of monocyte-derived dendritic cells. Res Immunol 149:627–632.
22. Rigby PW, Dieckmann M, Rhodes C, Berg P. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251.
23. Cotton M, Baker A, Saltik M, Wagner E, Buschle M. 1994. Lipopoly saccharide is a frequent contamination of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus. Gene Ther 1:239–246.
24. Hartmann G, Krieg AM. 1999. CpG DNA and LPS induce distinct patterns of activation in human monocytes. Gene Ther 6:893–903.
25. Okada C, Mizuochi T. 1999. Role of macrophages lysosomal enzymes in the degradation of nucleosomes of apoptotic cells. J Immunol 163:5346–5352.
26. Pearson AM, Rich A, Krieger M. 1993. Polynucleotide binding to macrophages scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. J Biol Chem 268:3546–3554.
27. Zardi L, Sirl A, Carnernolla B, Santi L. 1979. Fibronectin: A chromatin-associated protein? Cell 18:649–657.
28. Hornung V, Rothenfusser S, Britch S, Krug A, Jahrsdorfer B, Geise T, Endres S, Hartmann G. 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 168:4531–4537.
29. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 194:863–869.
30. Siren J, Pirhonen J, Julkunen I, Matikainen S. 2005. IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. J Immunol 174:1932–1937.
31. Spies B, Hochrein H, Vabulas M, Huster K, Busch DH, Schmitz F, Heit A, Wagner H. 2003. Vaccination with plasmid DNA activates dendritic cells via toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J Immunol 171:5908–5912.
32. Alton EW, Geddes DM, Gill DR, Higgins CF, Hyde SC, Innes JA, Porteous DJ. 1998. Towards gene therapy for cystic fibrosis: A clinical progress report. Gene Ther 5:291–292.
33. Freimark BD, Blezinger HP, Florack VJ, Nordstrom JL, Long SD, Deshpande DS, Nochumson S, Petrak KL. 1998. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: Cationic lipid complexes. J Immunol 160:4580–4586.
34. Paillard F. 1999. CpG: The double-edged sword. Hum Gene Ther 10:2089–2090.

IMMUNOLOGY ORIGINAL ARTICLE

DNA and its cationic lipid complexes induce CpG motif-dependent activation of murine dendritic cells

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doi:10.1111/j.1365-2567.2006.02451.x
Received 13 March 2006; revised 5 July 2006; accepted 5 July 2006.
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Summary

Unmethylated CpG motifs in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response of antigen-presenting cells (APC). In this study, we investigated the cytokine release from murine dendritic cells (DC) by the addition of various types of DNA in the free or complexed form with cationic lipids. Naked plasmid DNA and *Escherichia coli* DNA with immunostimulatory unmethylated CpG motifs induced pro-inflammatory cytokine secretion from granulocyte–macrophage colony-stimulating factor (GM-CSF)-cultured bone marrow-derived DC and the DC cell-line, DC2.4 cells, though vertebrate calf thymus DNA (CT DNA) with less CpG motifs did not. These characteristics differed from mouse peritoneal resident macrophages that do not respond to any naked DNA. The amount of cytokines released from the DC was significantly increased by complex formation with cationic lipids when CpG-motif positive DNAs were used. Unlike murine macrophages or Flt-3 L cultured DC, GM-CSF DC did not release inflammatory cytokines in response to the addition of CT DNA/cationic lipid complex, suggesting that the activation is completely dependent on CpG motifs. Taken together, the results of the present study demonstrate that murine DC produce pro-inflammatory cytokines upon stimulation with CpG-containing DNAs and the responses are enhanced by cationic lipids. These results also suggest that DC are the major cells that respond to naked CpG DNA *in vivo*, although both DC and macrophages will release inflammatory cytokines after the administration of a DNA/cationic lipid complex.

Keywords: CpG motifs; dendritic cells; TLR9; DNA and DNA uptake

Introduction

It is well known that unmethylated CpG sequences (CpG motifs) in bacterial DNA, but not in vertebrate DNA, are recognized by the immune system as a danger signal.[1] Cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12 and interferon-α (IFN-α) are secreted from antigen presenting cells, especially macrophages or dendritic cells (DC), upon stimulation with CpG DNA and synthetic oligodeoxynucleotides (ODN) containing CpG motifs. These cytokines significantly modify the therapeutic effects of DNA-based therapies in different ways.[2] For example, in gene therapy, cytokine production generally seems inappropriate because these inflammatory cytokines significantly reduce transgene expression in target cells through their direct

Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; BMDC, bone-marrow derived dendritic cell; CT DNA, calf thymus DNA; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; IL-12, interleukin-12; IFN-α, interferon-α; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IRF, interferon regulatory factor; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; MHC, major histocompatibility complex; TLR, Toll-like receptor; JNK, c-Jun NH_2-terminal kinase; Flt-3 L DC, Flt-3ligand cultured bone-marrow dendritic cells; EC DNA, *Escherichia coli* DNA, pDNA, plasmid DNA; FL-pDNA, fluorescein labelled plasmid DNA; GM-CSF, DC; granulocyte–macrophage, colony-stimulating factor cultured dendritic cells; DNase II, deoxyribonuclease II.

cytotoxicity and promoter attenuation.[3–5] On the other hand, it is essential for DNA vaccination because these cytokines can enhance the immune responses and profoundly affect the balance of these cytokines and the nature of the immune responses.[6–9]

DC are one of the most important cell populations as far as both innate and acquired immunity are concerned. They influence a variety of immunological responses associated with the therapeutic use of CpG DNA.[10,11] In addition to cytokine secretion, the expression of surface major histocompatibility complex (MHC) class I and II molecules as well as costimulatory molecules increases, and the maturation of DC is induced upon stimulation with CpG motifs.[12] The initial important step for all these processes associated with CpG DNA is cellular uptake because the receptor of CpG DNA, Toll-like receptor-9 (TLR9), is expressed within cells.[13,14] Our previous *in vitro* study using a DC cell line, DC2.4 cells, in mice demonstrated that DC take up pDNA via a mechanism specific to some defined polyanions[15] similar to cultured mouse peritoneal macrophages.[16,17]

There is a rapidly growing body of information about the mechanism of antigen-presenting cell (APC) activation by CpG DNA. This activation requires endosomal acidification and recognition by TLR9.[18–20] CpG DNA appears to use a TLR9 signaling pathway for NF-κB and c-Jun NH_2-terminal kinase (JNK) and IRF-7 through MyD88.[19,21] However, these proposed mechanisms are mainly based on studies using synthetic phosphorothioate CpG ODN, and there is little information about the activation induced by native DNA. Our previous study has demonstrated that, in contrast to macrophage cell lines, primary cultured mouse peritoneal macrophages secrete almost no inflammatory cytokines upon stimulation with pDNA, in spite of extensive uptake of the CpG DNA[22]. However, DNA/cationic lipid complex can activate the murine macrophages to induce inflammatory cytokines, whether they have replete CpG motifs or not.[23] Flt-3-ligand cultured bone-marrow DC (Flt-3 L DC) exhibit a different type of activation.[24,25] Upon stimulation with naked DNA, bacterial pDNA and CpG ODN stimulate Flt-3 L DC to induce cytokines IFN-α or IL-6 although vertebrate CT DNA does not. However, TLR9 in Flt-3 L DC can react when CT DNA is combined with cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP).[24] Methylated CpG motifs or non-canonical CpG motifs complexed with DOTAP induce the activation of TLR9 in Flt-3 L DC. Further experiments have proved that the other sequences also induce the activation of TLR9 when ODNs are translocated to endosomes by DOTAP.[25] While receptor-mediated endocytosis restricts the uptake of DNA, adsorptive endocytosis by cationic lipids does not. Thus, enhancement of DNA uptake seems to control the activation of TLR9 by vertebrate DNA. In the present study, we used a different type of DC and showed that the cells could respond to only DNA with CpG motifs even if the DNA was translocated to endosomes by cationic lipids.

Materials and methods

Chemicals

RPMI-1640 medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). *Escherichia coli* DNA (EC DNA) and calf thymus DNA (CT DNA) were purchased from Sigma (St Louis, MO). Lipofectin reagent and Opti-MEM were purchased from Invitrogen (Rockville, MD). Mouse recombinant GM-CSF (rGM-CSF) and Triton-X-114 were purchased from Nacalai Tesque (Kyoto, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Amersham, UK). Fetal Bovine Serum (FBS) was purchased from Thermo Trace (Melbourne, Australia).

Cell culture

Male ICR mice (5 weeks) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). After bone marrow was flushed out of the bones of the hind legs of the mice, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml rGM-CSF. After a 4–5 day incubation at 37° in 5% CO_2-95% air, cells were collected and centrifuged at 200 *g* for 10 min. After removal of the supernatant, the cells were resuspended in 400 μl phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA) per 10^8 total cells. The cell suspension was mixed thoroughly with 100 μl magnetic-activated cell sorting (MACS) CD11c MicroBeads (Miltenyi Biotec, Germany), and incubated for 15 min at 4°. After incubation, the cells were washed, centrifuged at 200 *g* for 10 min, and resuspended in 500 μl PBS containing 0·5% BSA. Then, magnetic separation with MACS was carried out to isolate the DC by selecting CD11c-positive cells from the cultured cells. These isolated cells were washed and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well and cultured for 24 hr. The murine DC2.4 cells were a gift from Dr Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA). DC2.4 cells display dendritic morphology, express dendritic cell-specific markers, MHC molecules, and costimulatory molecules, and exhibit phagocytic activity and an antigen-presenting capacity.[26] DC2.4 cells were cultured with RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 50 μM 2-mercaptoethanol, and antibiotics. They were then plated on a 24-well culture plate at a density of 5×10^5 cells/well and cultured for 24 hr.

DNA

pCMV-Luc encoding firefly luciferase gene was constructed, as described previously.[27] pDNA was purified using an Endo-free plasmid Giga kit (Qiagen, Valencia, CA). For the cellular association experiments, pDNA was radiolabelled with [α-^{32}P]dCTP by nick translation.[28] For the activation experiments, all DNA samples were extensively purified with Triton-X-114, a non-ionic detergent, to minimize the activation by contaminated lipopolysaccharide (LPS). Extraction of endotoxin from pDNA, EC DNA, and CT DNA samples was performed according to reviously published methods[29,30] with slight modifications. DNA samples were purified by extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and ethanol precipitation. Then, 10 mg DNA was diluted with 20 ml pyrogen-free water, followed by the addition of 200 μl Triton-X-114 and mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55°. Subsequently, the solution was centrifuged for 20 min at 25°, 600 *g*. The upper phase was transferred to a new tube, 200 μl Triton-X-114 was added, and the previous steps were repeated at least three times. The activity of LPS was measured by *Limulus* amoebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free plasmid Giga kit, 1 μg/ml pDNA contained 0·01–0·05 EU/ml endotoxin. After Triton-X-114 extraction, the endotoxin levels of the 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, which contains 1–5 EU/ml endotoxin, could release 521 ± 73 pg/ml TNF-α at 24 hr from peritoneal macrophages.

Cationic liposome formation

Lipofectin complexes were prepared according to the manufacturer's instructions. In brief, DNA was diluted in 100 μl Opti-MEM per 1 μg DNA (solution A) and 5 μl Lipofectin reagent was diluted in another 100 μl Opti-MEM (solution B). Then solutions A and B were combined and mixed gently. After a 15 min incubation at room temperature, complex was added to the cells.

Cellular association experiments

DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml Hanks' balanced salt solution (HBSS) without phenol red and 0·5 ml HBSS containing 0·1 μg/ml naked [^{32}P]pDNA or 0·1 μg/ml [^{32}P]pDNA/Lipofectin complex was added. After incubation at 37 or 4° for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1·0 ml 0·3 N NaOH with 0·1% Triton-X-100. Aliquots of the cell lysate were taken for the determination of ^{32}P radioactivity using an LSA-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content was measured using the modified Lowry method with BSA as a standard.

Confocal microscopy

DC2.4 cells were washed three times with 1·0 ml HBSS and incubated with HBSS containing fluorescein-labelled pDNA (FL-pDNA) or FL-pDNA/Lipofectin complex. After a 3 hr incubation, the cells were washed five times and fixed with 4% paraformaldehyde for 10 min.

Cytokine secretion

BMDC or DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml RPMI-1640 before use. Naked DNA was diluted in 0·5 ml Opti-MEM. The cells were incubated with the naked DNA solution continuously for 8 hr. In the case of DNA/Lipofectin complexes, cells were incubated for 2 hr with 0·5 ml of the solutions containing the complexes. Then, the cells were washed with RPMI-1640 and incubated with RPMI-1640 with 10% FBS. After a 6 hr incubation, the supernatant was collected for ELISA and kept at −80°. The levels of TNF-α, IL-6, and IL-12p70 in the supernatants were determined by the OptEIA Set (BD Biosciences, San Diego, CA).

Results

Uptake of DNA with cationic lipid complexes is not saturated, although normal uptake is saturated in GM-CSF DC

TLR9 exists in the endosomal-lysosomal compartment.[13,14] The amount of naked DNA in the compartment can be limited because naked DNA is supposed to be taken up by DC via receptor-mediated endocytosis.[15] However, DNA/cationic lipid complexes are supposed to be taken up by DC via a non-specific mechanism based on electrostatic interaction, so-called adsorptive endocytosis. Therefore, cationic lipid Lipofectin was used to deliver DNA efficiently to the compartment. To examine the binding and uptake of naked pDNA and pDNA/cationic lipid complexes in DC, we carried out cellular uptake experiments using naked [^{32}P]pDNA and [^{32}P]pDNA/Lipofectin complexes. As expected, the uptake of naked [^{32}P]pDNA by DC2.4 cells at 37° was increased up to 2 hr (Fig. 1a). Following an incubation of 2–5 hr, the amount of DNA remained unchanged, probably due to continued uptake and degradation.[15] On the other hand, complexation with cationic lipids enhanced the DNA uptake. Cationic lipids enhanced [^{32}P]pDNA binding and

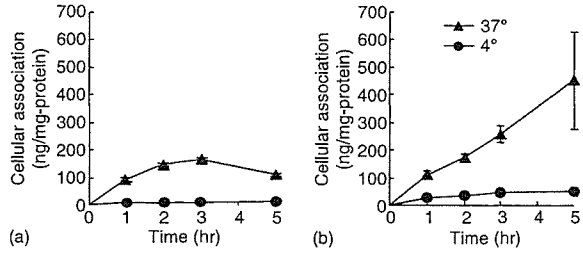

Figure 1. Cellular association time courses of naked [^{32}P]pDNA (a) or [^{32}P]pDNA/Lipofectin complex (b) in DC2.4 cells. Cells were incubated at 37° (closed triangle) or 4° (closed circle). Each point represents the mean ± SD (n = 3).

uptake in DC2.4 cells and the amount of [^{32}P]pDNA increased in a time-dependent manner (Fig. 1b).

Next, we examined the localization of fluorescence-labelled DNA (FL-pDNA). In the confocal microscopy experiments, the fluorescence derived from naked FL-pDNA is bound to the cellular membrane at 4° (Fig. 2a). At 37°, FL-pDNA was observed inside the cells after 1 hr and it appeared to accumulate in the nucleus after a 3 hr incubation. On the other hand cationic lipids completely changed the localization of DNA. The fluorescence of the FL-pDNA/Lipofectin complex was observed in a punctuated pattern at 1 hr, then diffused into the cells after a 3 hr incubation (Fig. 2b).

The activation of GM-CSF DC by DNA

Next, cytokine production from DC by naked DNA was examined. Plasmid DNA and *E. coli* DNA were used as models of bacterial CpG DNA, and calf thymus DNA was used as a model of vertebrate DNA. As shown in Fig. 3, naked bacterial plasmid DNA and *E. coli* DNA with replete immunostimulatory CpG motifs induced TNF-α, IL-6 and IL-12 secretions from bone marrow-derived DC.

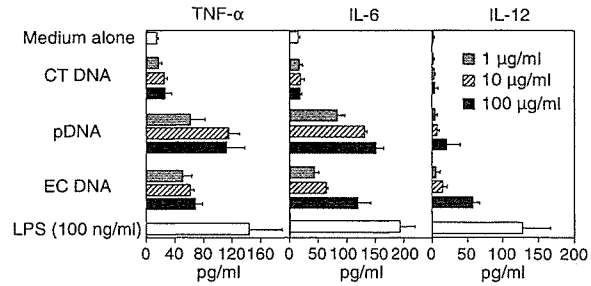

Figure 3. Cytokine secretion induced by naked DNA from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

The results are consistent with previous studies demonstrating that plasmid DNA stimulates GM-CSF DC to induce TNF-α and IL-12.[18] Vertebrate calf thymus DNA (CT DNA) containing less CpG motifs did not. LPS induced small amounts of cytokines, probably because of relatively short-term incubation (8 hr). Similar results were observed in the experiment using DC2.4 cells, although the cells released a higher amount of cytokines (Fig. 4). These results demonstrate that the cytokine secretion from the DC corresponds to the difference between endogenous DNA and exogenous DNA.

Next, cellular activation in DC by DNA/cationic lipid complexes was examined. The *E. coli* DNA/Lipofectin complexes stimulated GM-CSF cultured DC to produce cytokines, TNF-α, IL-6 and IL-12 in a dose-dependent manner (Fig. 5). Similar results were observed with pDNA/Lipofectin complex. The amounts of cytokines released from the DC were significantly increased by complex formation with cationic lipids compared with naked DNA (Fig. 3). The DC were unable to produce a significant amount of pro-inflammatory cytokines following

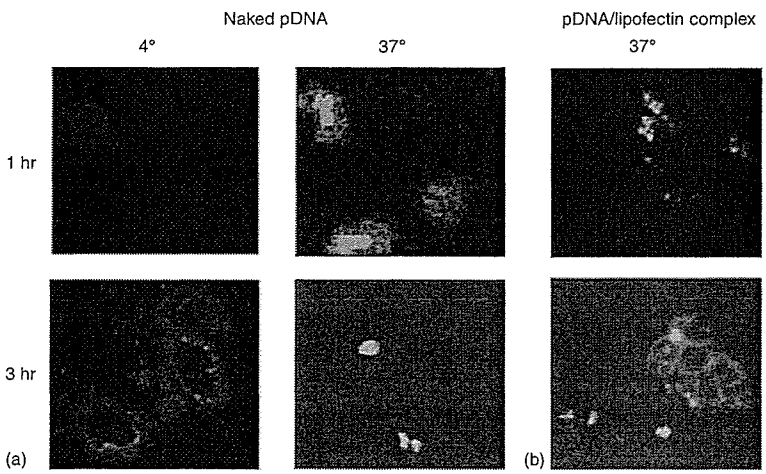

Figure 2. Uptake of naked FL-pDNA (a) or FL-pDNA/Lipofectin complex (b) by DC2.4 cells. The cells were incubated with 5·0 μg/ml naked FL-pDNA or 30 μg/ml FL-pDNA/Lipofectin complex.

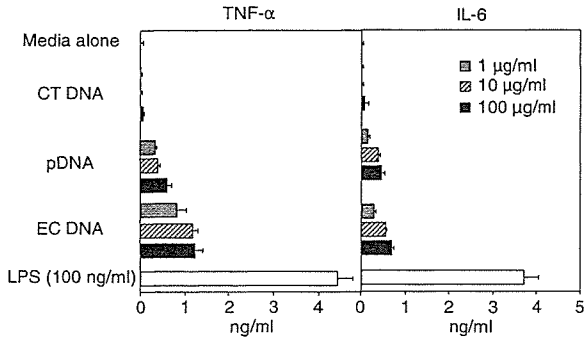

Figure 4. Cytokine secretion induced by naked DNA from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

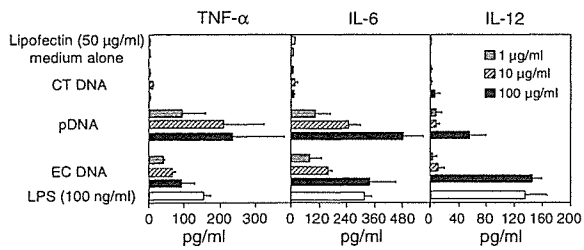

Figure 5. Cytokine secretion induced by DNA/Lipofectin complex from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 hr after the incubation with liposomes. The amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

stimulation with vertebrate calf thymus DNA (CT DNA) containing less CpG motifs when DNA is complexed to Lipofectin. Lipids alone were unable to stimulate the DC sufficiently to release pro-inflammatory cytokines. Similar results were obtained in DC2.4 cells (Fig. 6). These results demonstrate that GM-CSF DC discriminate between bacterial DNA and mammalian DNA.

Discussion

The most important role of immune system is to distinguish between 'self' and 'non-self'. Although the TLR9 subfamily (TLR7, 8 and 9) recognizes non-self nucleic acids[31] under special conditions, such as systemic lupus erythematosus, these TLRs are stimulated in response to self nucleic acids. For example chromatin–immunoglobulin complexes trigger DC activation in a TLR9-dependent and TLR9-independent manner.[32] Recently, Barton *et al.* have demonstrated that the fusion protein of

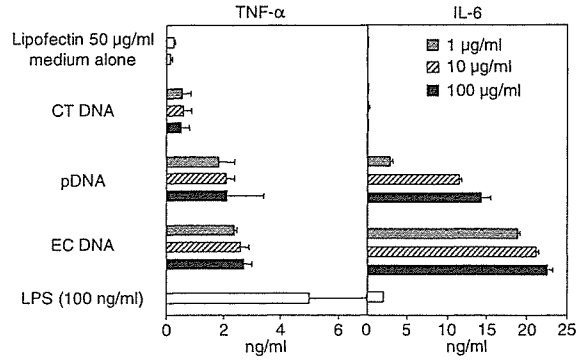

Figure 6. Cytokine secretion induced by DNA/Lipofectin complex from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after the incubation with liposomes. The amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

TLR4/9, which is delivered to cellular membranes, is activated by vertebrate DNA.[33] One proposed hypothesis is that compartmentalization of TLR9 prevents the response induced by endogenous DNA.

In the present study, we have demonstrated that GM-CSF-derived DC activation is triggered by exogenous naked DNA. Bacterial DNA induces cytokine secretion from DC, although vertebrate DNA does not. Flt-3 L cultured murine DC (Flt-3 L DC) also induce activation of TLR9 in response to naked bacterial DNA, but not naked vertebrate DNA.[24] Therefore, these studies imply that both GMCSF-DC and Flt-3 L DC can discriminate between bacterial non-self DNA and vertebrate self DNA.

On the other hand, these characteristics are different from murine macrophages.[22] Primary macrophages do not respond to naked DNA in spite of TLR9 expression, although the macrophage-like cell line RAW264.7 cells do. Both primary macrophages and DC take up DNA via a similar mechanism.[15–17] The mechanism of unresponsiveness of macrophages to DNA has not been elucidated, although TLR9 is present in the cells. Macrophages have deoxyribonuclease II (DNase II) in the lysosomal compartment, and they are responsible for apoptotic cell engulfment, DNA digestion and erythroid cell differentiation.[34] In erythropoiesis, macrophages take up nuclei and digest DNA. In DNase II-deficient mice, undigested DNA in macrophages causes IFN-β production via unknown receptors.[35] The cytokine production is mediated by the TLR9/MyD88 pathway and novel pathways that have been identified recently.[36,37] Therefore, the mechanism of the unresponsiveness of macrophages to naked DNA may involve the limited uptake and degradation by DNase II. However, further investigation is required.

The TLR4/9 fusion protein on the cell membrane is activated by vertebrate DNA.[33] This research indicates that compartmentalization into cells avoids TLR9 responses to endogenous DNA. Therefore, we forced DNA to internalize into cells using cationic lipids. In fact, vertebrate DNA/cationic lipid complexes can induce cytokine secretion from murine macrophages and Flt-3 L DC.[23,24] Following enhancement of DNA uptake by cationic lipids, these cells cannot distinguish between 'self' and 'non-self' DNA. In peritoneal macrophages, complexation of calf thymus DNA with cationic lipids elicited a similar level of inflammatory cytokine production to that obtained with bacterial *E. coli* DNA using cationic lipids.[23] In addition, calf thymus DNA with cationic lipid DOTAP causes a high degree of IFN-α release from murine Flt-3 L cultured DC or human peripheral blood mononuclear cells.[24] The amount of IFN-α induced by calf thymus DNA with DOTAP is similar to that induced by bacterial plasmid DNA. However, the result with GM-CSF DC is different from that in these cells. The cells only recognize bacterial DNA. Vertebrate DNA/cationic lipid complexes do not stimulate GM-CSF DC, although bacterial DNA does. There are two possibilities to explain these observations. One is the possibility that different types of cationic lipids lead to different forms of delivery of DNA, and result in different responses. For example, murine macrophages release inflammatory cytokines in response to the addition of vertebrate CT DNA/cationic lipid complexes.[23] Lipofectamine was used for this research. Synthetic double-stranded DNA containing no CpG motif can stimulate macrophage cell lines when DNA is complexed with the cationic lipid Fugene 6.[38] In addition, vertebrate CT DNA/cationic lipid Lipofectamine complexes induce macrophage activation via TLR9-dependent and -independent mechanisms.[39] Flt-3 L cultured DC (Flt-3 L DC) also responds to vertebrate DNA/cationic lipid DOTAP complexes via TLR9-dependent and -independent pathways.[24] TLR9-independent activation is also observed following transfection using Lipofectamine 2000.[37] Honda *et al.* showed that different cellular distributions of DNA result in different cytokine responses.[40] CpG-B ODN normally do not induce IFN-α release from plasmacytoid DC. However, following complexation with DOTAP, the same ODNs trigger IFN-α. Confocal microscopy reveals that DOTAP retains DNA in early endosomes, although ODNs without DOTAP are immediately transferred to lysosomal vesicles. Taken together, enhancement of the DNA uptake may not explain the response of TLR9 to vertebrate DNA and TLR9 may be present in specific compartments.

The other possibility is that GM-CSF DC, Flt-3 L DC and macrophages may contribute to the immune systems in different ways, by producing different types or degrees of induction. TLR9 is mainly expressed in B cells and plasmacytoid DC in humans.[31] On the other hand, mouse TLR9 is also present in myeloid DC and macrophages. Although further studies are required to clarify the contribution of DC or macrophages to immune responses *in vivo*, the present study suggests that DC are the main cells that respond to naked bacterial DNA, although both DC and macrophages will release inflammatory cytokines after the administration of bacterial DNA/cationic lipid complexes.

Very recently Martin *et al.* have shown that GM-CSF DC release type I IFN upon stimulation of mammalian DNA complexed with Fugene, another kind of lipid for transfection.[41] Interestingly, the cells do not produce TNF-α, IL-6 or IL-12. The activation is independent of TLR9 because GM-CSF DC from TLR9$^{-/-}$ deficient mice respond to mammalian DNA/Fugene complexes to secrete type I IFN. Another group has also demonstrated that non-CpG DNA/lipofectamine complexes stimulate GM-CSF DC to induce type I IFN.[42] The activation is not dependent on the MyD88 or TRIF pathways. Based on these observations, one can hypothesize that, distinct from Flt-3 L DC, GM-CSF DC respond to only bacterial or viral DNA via TLR9-dependent pathway, and release cytokines, such as TNF-α IL-6 and IL-12. However when mammalian DNAs are translocated into cells, GM-CSF DC may not induce these cytokines. Instead, the cells may release IFN-α through a TLR9-independent pathway. Further studies are required for these TLR9-dependent and -independent mechanisms.

In conclusion, the present study has demonstrated that murine GM-CSF DC or the DC cell line, DC2.4, produce pro-inflammatory cytokines following stimulation with CpG-containing DNAs and this production is increased when the DNAs are added to the cells in a complex form with cationic lipids. These findings form an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

Acknowledgements

This work was supported in part by 21st Century COE Program 'Knowledge Information Infrastructure for Genome Science', and also in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We would like to thank Dr Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA, USA) for providing DC2.4 cells.

References

1 Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002; 20:709–60.

2 Yasuda K, Wagner H, Takakura Y. Role of immunostimulatory DNA and TLR9 in gene therapy. *Crit Rev Ther Drug Carrier Syst* 2006; 23:89–110.

3 Haddad EB, Rousell J, Lindsay MA, Barnes PJ. Synergy between tumor necrosis factor alpha and interleukin 1beta in inducing transcriptional down-regulation of muscarinic M2 receptor gene expression. Involvement of protein kinase A and ceramide pathways. *J Biol Chem* 1996; 271:32586–92.

4 Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy. interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997; 8:2019–29.

5 Sellins K, Fradkin L, Liggitt D, Dow S. Type I Interferons potently suppress gene expression following gene delivery using liposome (–) DNA complexes. *Mol Ther* 2005; 12:451–9.

6 Gurunathan S, Klinman DM, Seder RADNA vaccines: immunology, application, and optimization. *Annu Rev Immunol* 2000; 18:927–74.

7 Raz E, Tighe H, Sato Y *et al.* Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996; 93:5141–5.

8 Roman M, Martin OE, Goodman JS *et al.* Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997; 3:849–54.

9 Seder RA, Hill AV. Vaccines against intracellular infections requiring cellular immunity. *Nature* 2000; 406:793–8.

10 Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. *Int J Cancer* 2001; 94:459–73.

11 Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c (–) type 2 dendritic cell precursors and CD11c (+) dendritic cells to produce type I IFN. *J Immunol* 2001; 166:2291–5.

12 Weiner GJ. The immunobiology and clinical potential of immunostimulatory CpG oligodeoxynucleotides. *J Leukoc Biol* 2000; 68:455–63.

13 Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol* 2002; 32:1958–68.

14 Latz E, Schoenemeyer A, Visintin A *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 2004; 5:190–8.

15 Yoshinaga T, Yasuda K, Ogawa Y, Takakura Y. Efficient uptake and rapid degradation of plasmid DNA by murine dendritic cells via a specific mechanism. *Biochem Biophys Res Commun* 2002; 299:389–94.

16 Takagi T, Hashiguchi M, Mahato RI, Tokuda H, Takakura Y, Hashida M. Involvement of specific mechanism in plasmid DNA uptake by mouse peritoneal macrophages. *Biochem Biophys Res Commun* 1998; 245:729–33.

17 Takakura Y, Takagi T, Hashiguchi M *et al.* Characterization of plasmid DNA binding and uptake by peritoneal macrophages from class A scavenger receptor knockout mice. *Pharm Res* 1999; 16:503–8.

18 Hacker H, Mischak M, Miethke T *et al.* CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J* 1998; 17:6230–40.

19 Yi AK, Tuetken R, Redford T, Waldschmidt M, Kirsch J, Krieg AM. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J Immunol* 1998; 160:4755–61.

20 Hemmi H, Takeuchi O, Kawai T *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; 408:740–5.

21 Honda K, Yanai H, Mizutani T *et al.* Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci USA* 2004; 101:15416–21.

22 Yasuda K, Kawano H, Yamane I, Ogawa Y, Yoshinaga T, Nishikawa M, Takakura Y. Restricted cytokine production from mouse peritoneal macrophages in culture in spite of extensive uptake of plasmid DNA. *Immunology* 2004; 111:282–90.

23 Yasuda K, Ogawa Y, Kishimoto M, Takagi T, Hashida M, Takakura Y. Plasmid DNA activates murine macrophages to induce inflammatory cytokines in a CpG motif-independent manner by complex formation with cationic liposomes. *Biochem Biophys Res Commun* 2002; 293:344–8.

24 Yasuda KYuP, Kirschning CJ *et al.* Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. *J Immunol* 2005; 174:6129–36.

25 Yasuda K, Rutz M, Schlatter B *et al.* CpG motif-independent activation of TLR9 upon endosomal translocation of 'natural' phosphodiester DNA. *Eur J Immunol* 2006; 36:431–6.

26 Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* 1997; 158:2723–30.

27 Nomura T, Yasuda K, Yamada T, Okamoto S, Mahato RI, Watanabe Y, Takakura Y, Hashida M. Gene expression and antitumor effects following direct interferon (IFN)-gamma gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice. *Gene Ther* 1999; 6:121–9.

28 Sambrook J, Fritsch EF, Maniatis T *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring. Harbor Laboratory Press, 1989.

29 Cotten M, Baker A, Saltik M, Wagner E, Buschle M. Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus. *Gene Ther* 1994; 1:239–46.

30 Hartmann G, Krieg AM. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Ther* 1999; 6:893–903.

31 Wagner H. The immunobiology of the TLR9 subfamily. *Trends Immunol* 2004; 25:381–6.

32 Boule MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med* 2004; 199:1631–40.

33 Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol* 2006; 7:49–56.

34 Nagata S. DNA degradation in development and programmed cell death. *Annu Rev Immunol* 2005; 23:853–75.

35 Yoshida H, Okabe Y, Kawane K, Fukuyama H, Nagata S. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat Immunol* 2005; 6:49–56.

36 Okabe Y, Kawane K, Akira S, Taniguchi T, Nagata S. Toll-like receptor-independent gene induction program activated by

mammalian DNA escaped from apoptotic DNA degradation. *J Exp Med* 2005; **202**:1333–9.

37 Ishii KJ, Coban C, Kato H *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* 2006; **7**:40–8.

38 Zhu FG, Reich CF, Pisetsky DS. Effect of cytofectins on the immune response of murine macrophages to mammalian DNA. *Immunology* 2003; **109**:255–62.

39 Yasuda K, Ogawa Y, Yamane I, Nishikawa M, Takakura Y. Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways. *J Leukoc Biol* 2005; **77**:71–9.

40 Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T. Spatiotemporal regulation of MyD88-IRF-7 signaling for robust type-I interferon induction. *Nature* 2005; **434**:1035–40.

41 Martin DA, Elkon KB. Intracellular mammalian DNA stimulates myeloid dendritic cells to produce type I interferons predominantly through a toll-like receptor 9-independent pathway. *Arthritis Rheum* 2006; **54**:951–60.

42 Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 2006; **24**:93–103.