Cellular Activation by Plasmid DNA in Various Macrophages in Primary Culture

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ABSTRACT: Macrophages are an important group of cells responsible for the inflammatory response to unmethylated CpG dinucleotide (CpG motif) in plasmid DNA (pDNA) via Toll-like receptor 9 (TLR9). This finding is primarily based on *in vitro* studies. Previous *in vivo* studies also have suggested that tissue macrophages are involved in inflammatory cytokine release in the circulation following intravenous administration of pDNA to mice. However, the relationship between the *in vitro* and *in vivo* studies has not been sufficiently clarified. To gain insight into which types of cells are responsible for the production of cytokines upon interaction with pDNA, peritoneal macrophages, splenic macrophages, hepatic nonparenchymal cells (NPCs) including Kupffer cells and mesangial cells were isolated from mice. All types of primary cultured cells, except for mesangial cells, express TLR9 at varying levels. Splenic macrophages and hepatic NPCs were activated to produce tumor necrosis factor-α (TNF-α) by naked pDNA, whereas peritoneal macrophages and mesangial cells were not. pDNA complexed with *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride/cholesterol liposome induced TNF-α in the splenic macrophages but not in the other cell types. These results indicate that splenic macrophages and hepatic NPCs are closely involved in TNF-α production in response to pDNA. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:4575–4585, 2008

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INTRODUCTION

Plasmid DNA (pDNA) has become an important macromolecular agent suitable for nonviral gene therapy as well as DNA vaccination.[1] It is well known that unmethylated CpG dinucleotides, or CpG motifs, in bacterial DNA, but not in vertebrate DNA, are recognized by the mammalian immune system as a danger signal.[2] Toll-like receptor 9 (TLR9) is a pattern recognition receptor recognizing CpG motifs,[3] and is present in the intracellular compartments of immune cells, such as macrophages and dendritic cells.[4] These cells secrete inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), and IL-12, upon uptake of CpG motif-containing DNA. These cytokines reduce transgene expression in target cells through direct cytotoxicity and/or promoter attenuation.[5,6] Consequently, reduction or prevention of pDNA-induced cytokine production is important for increasing the efficiency of *in vivo* gene transfer.

To improve the transfection efficacy with pDNA, DNA/cationic liposome complexes are often used both *in vivo* and *in vitro*. Several

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recent studies have shown that intravenous (i.v.) administration of pDNA/cationic liposome complexes leads to effective gene expression although such complexes also induce high amounts of inflammatory cytokines.[5–10]

In a series of investigations on the tissue disposition of naked pDNA we have shown that the hepatic nonparenchymal cells (NPCs), such as Kupffer cells and sinusoidal endothelial cells, play important roles in the clearance of pDNA.[11,12] In addition, it has been suggested that tissue macrophages, such as Kupffer cells (liver resident macrophages) and splenic macrophages, are responsible for cytokine production following intravenous injection of DNA/cationic liposome complexes.[13] On the other hand, there is very little production of cytokines by cultured peritoneal macrophages following stimulation with naked pDNA,[14] even although they exhibit extensive DNA uptake via scavenger receptorlike mechanisms.[15,16] Surprisingly, peritoneal macrophages produce inflammatory cytokines upon incubation with DNA/cationic liposome complexes irrespective of the presence of the CpG motif.[17] Moreover, we found that this response was induced via a TLR9-independent pathway, because primary cultured cells from TLR9 knockout mice also released cytokines upon incubation with such complexes.[18,19]

In spite of extensive studies on the cellular activation induced by CpG DNA including pDNA, the relationship between *in vitro* and *in vivo* studies requires further investigation because specific types of immune cells including cell lines were generally used in these *in vitro* studies. In order to understand the pDNA-mediated cellular activation *in vivo*, it is necessary to evaluate the capability of various types of macrophages and macrophage-like cells to induce inflammatory cytokines because pDNA is distributed to some organs after i.v. administration. In the present study, we examined whether primary cultured cells from mouse organs, including peritoneal macrophages, are activated by naked DNA or DNA/cationic liposome complexes in order to identify the roles of these cells in the immune response at the whole-body level.

MATERIALS AND METHODS

Chemicals

N-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol and Triton X-114 was purchased from Nacalai Tesque (Kyoto, Japan). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS), a cocktail of protease inhibitors, calf thymus (CT) DNA and type I-A collagenase were purchased from Sigma (St. Louis, MO). ITS^{TM} was purchased from BD Bioscience (San Diego, CA). Monoclonal mouse anti-mouse TLR9 antibody was purchased from InvivoGen (San Diego, CA) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was purchased from Amersham Biosciences Inc. (Piscataway, NJ).

Plasmid DNA

Plasmid vector pCMV-Luc, which encodes firefly *luciferase* gene, was constructed based on pcDNA3 as described previously.[20] pCMV-Luc has 33 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT, one of the most potent CpG motifs for mice.[21] pCMV-Luc was amplified in the *E. coli* strain DH5α and then isolated and purified using a Qiagen Endofree^{TM} Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany).

Purification of DNA

To minimize the activation by contaminated LPS, DNA samples were extensively purified with Triton X-114, a nonionic detergent. Extraction of LPS from pDNA and CT DNA samples was performed according to previously published methods.[14,22,23] The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free^{TM} plasmid Giga kit, the level of LPS was found to be 0.01–0.05 EU/μg pDNA, which was reduced below the detection limit of 0.001 EU/μg pDNA by the Triton X-114 extraction.

Preparation of Cationic Liposomes and DNA/Cationic Liposome Complexes

In the present study, DOTMA/cholesterol liposomes were used to prepare the DNA complexes because these complexes are widely used in *in vivo* studies due to their high *in vivo* transfection efficacy.[24,25] Cationic liposomes consisting of DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin

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