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Involvement of Specific Mechanism in Plasmid DNA Uptake by Mouse Peritoneal Macrophages

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The binding and uptake of plasmid DNA encoding luciferase reporter gene (pCMV-Luc) were studied in vitro using cultured mouse peritoneal macrophages. A significant and time-dependent cellular association of [^{32}P]pCMV-Luc with resident macrophages was observed at 37 °C and this decreased at 4 °C. The binding at 4 °C was saturable and a Scatchard plot gave a maximum binding capacity of 0.81 μg/mg-protein and a dissociation constant of 0.30 μg/ml. The binding of [^{32}P]-pCMV-Luc was inhibited by polyinosinic acid, dextran sulfate and salmon sperm DNA, but not by polycytidylic acid, dextran and EDTA. A confocal microscopic study demonstrated that fluorescein-labeled pCMV-Luc was internalized at 37 °C while only cell surface binding occurred at 4 °C. No significant luciferase gene expression was obtained after incubation with a high concentration (100 μg/ml) of pCMV-Luc. These data suggest that plasmid DNA is taken up by macrophages via a mechanism mediated by a receptor like the macrophage scavenger receptor. © 1998 Academic Press

Key Words: **mouse peritoneal macrophages; plasmid DNA; receptor-mediated endocytosis; scavenger receptor.**

Recently, plasmid DNA has become an important class of macromolecular therapeutic agents for non-viral gene therapy (1,2) and DNA vaccination (3,4). In vivo use of plasmids is thought to be safer than that of viral vectors or attenuated viruses because there is less potential for adverse effects. Administration of large quantities or repeated dosing of naked plasmid DNA is often achieved by systemic or local injection.

On the other hand, there is increasing evidence suggesting that bacterial DNA activates immune competent cells, especially macrophages (5-8). For example, bacterial DNA is reportedly taken up by macrophages (5) and activates inflammatory gene induction such as tumor necrosis factor (TNF)-α (5) and interleukin 12 (6). Moreover, lethal toxic shock can be evoked by induction of macrophage-derived TNF-α after administration of bacterial DNA in mice (7,8). These phenomena are not induced by mammalian DNA. However, the cellular uptake mechanism of plasmid DNA is poorly understood.

In a previous study, we have shown that naked plasmid DNA is rapidly taken up by the liver, preferentially by the non-parenchymal liver cells, after intravenous injection in mice (9). The hepatic uptake is significantly inhibited by preceding administration of typical ligands of the macrophage scavenger receptor, such as polyinosinic acid, dextran sulfate and maleylated bovine serum albumin, but not by polycytidylic acid (9). Identical results have been obtained in perfusion experiments on plasmid DNA using perfused rat liver (10). These results suggest that hepatic uptake of plasmid DNA is predominantly mediated by macrophage scavenger receptors expressed on liver macrophages, Kupffer cells, which recognize a wide variety of anionic macromolecules based on their three dimensional structure (11-13).

In the present study, we examined the binding and uptake of plasmid DNA in cultured mouse peritoneal macrophages to investigate the uptake mechanism. Here, we report that macrophages take up plasmid DNA by a mechanism mediated by a receptor similar to the scavenger receptor.

MATERIALS AND METHODS

Chemicals. Thioglycolate (TGC) broth, RPMI 1640 medium and Ham's F-12 medium were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-^{32}P]dCPT (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). Polyinosinic acid (poly[I],

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M.W. 103.3 kDa), polycytidylic acid (poly[C], M.W. 99.5 kDa), dextran (M.W. 70 kDa) were purchased from Pharmacia (Uppsala, Sweden). Dextran sulfate (M.W. 150 kDa) was purchased from Nacalai Tesque (Kyoto, Japan). pGL3 control vector and pCAT (plasmid DNA encoding chloramphenicol acetyltransferase reporter gene) control vector were purchased from Promega (Madison, WI). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD). Ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt (EDTA.2Na) was purchased from Dojindo (Kumamoto, Japan). All other chemicals used were of the highest purity available.

Preparation of plasmid DNA. pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector into the polylinker of pcDNA3 vector. pCMV-Luc and pCAT were amplified in the DH5α and HB101 strains of Escherichia coli, respectively. Plasmids were extracted and purified by QIAGEN Plasmid Giga Kit and EndoFree Plasmid Buffer Set (QIAGEN GmbH, Hilden, Germany), and diluted with sterilized saline. Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm. For cellular association experiments, pCMV-Luc and pCAT were radiolabeled using [α-^{32}P]dCPT and T4 polynucleotide kinase, as described by Sambrook et al. (14). For the confocal microscopic study, pCMV-Luc was labeled with fluorescein using a FastTag FL labeling kit (Vector Laboratories, Burlingame, CA).

Harvesting and culture of macrophages. Male ICR mice (20-25 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Resident macrophages were collected from the peritoneal cavity of unstimulated mice with RPMI 1640 medium. Washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) and plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well for the uptake experiment, or plated on 35 mm glass bottom microwell dishes (MatTek, Ashland, MA) for confocal microscopic observation. After incubation for 2 hr at 37 °C in 5% CO_2-95% air, adherent macrophages were washed three times with RPMI 1640 medium to remove nonadherent cells and then cultured under the same conditions for 24 hr. For elicited macrophages, all the processes were the same, except that 1 ml 2.9% thioglycolate broth was injected intraperitoneally into mice 4 days prior to macrophage collection. Chinese hamster ovary (CHO-K1) cells obtained from Dainippon Pharmaceutical were used as a control. The CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml).

Cellular association experiments. Mouse peritoneal macrophages or CHO-K1 cells were cultured in 24-well plates. The cells were washed three times with 0.5 ml Hanks' balanced salt solution (HBSS) without phenol red, and 0.5 ml HBSS containing [^{32}P]pCMV-Luc or [^{32}P]pCAT (0.1 μg/ml) was added. After incubation at 37 °C or 4 °C for a specified time, the cells were washed three times with 0.5 ml ice-cold HBSS and then solubilized with 1.0 ml 0.3 N NaOH with 0.1% Triton X-100. Aliquots were taken for the determination of ^{32}P radioactivity using an LSA-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content measured using the modified Lowry method (15) with bovine serum albumin as a standard. To examine the competition in binding and uptake, unlabeled macromolecules such as poly[I], poly[C], dextran, and dextran sulfate were added to the incubation wells concomitant with [^{32}P]pCMV-Luc or [^{32}P]pCAT. The incubation was continued at 4 °C for 3 hr, and the radioactivity and protein content were determined as described above. In the binding experiment at 4 °C, non-specific binding was defined as the binding in the presence of 50 μg/ml unlabeled plasmid DNA and subtracted from the total binding to calculate the specific binding. The non-specific binding ranged from 3 to 10% of the total binding (non-specific binding plus specific binding).

Confocal microscopic study. Resident macrophages were washed three times with 1.5 ml HBSS, and 100 μl HBSS containing fluorescein-labeled pCMV-Luc ([Fl]pCMV-Luc, 5.0 μg/ml) was added. After 3 hr incubation, the cells were washed five times with 1.5 ml ice-cold HBSS and fixed with 10% neutral formalin buffer for 30 min. Then, the cells were washed three times with 1.5 ml HBSS, and scanned with a confocal laser microscope (ACAS 570, Meridian Instruments, Okemos, MI).

Gene expression experiments. The transfection potency of naked pCMV-Luc was examined using resident macrophages cultured in a 6-well plate. The cells were first rinsed twice with PBS to remove serum, and serum-free Opti-MEM I medium (1.0 ml) containing naked plasmid DNA was added to each well. The transfection solution was removed after 6 hr and replaced with fresh medium containing FBS. Two days after transfection, the cells were washed with PBS and harvested by scraping and transferred to Eppendorf tubes and subjected to luciferase assay. The light units produced were measured on a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany).

RESULTS

A significant and time-dependent cellular association of [^{32}P]pCMV-Luc with resident macrophages was observed at 37 °C and this decreased at 4 °C (Fig. 1A). In elicited macrophages, however, the association was markedly reduced (Fig. 1B). The cellular association of [^{32}P]pCMV-Luc with CHO-K1 cells at both temperatures was significantly lower than that observed in the resident macrophages (Fig. 1C). The binding of [^{32}P]-pCMV-Luc to the resident macrophages at 4 °C was saturable (Fig. 2A) A Scatchard analysis of this binding data gave a straight line indicating a single homogeneous population of binding sites with a maximum binding (B_{max}) of 0.81 μg/mg-protein and a dissociation constant (K_D) of 0.30 μg/ml (Fig. 2B).

The specificity of the binding of [32P]pCMV-Luc was examined by cross-competition experiments using various macromolecules. As shown in Fig. 3, excess amounts of unlabeled pCMV-Luc inhibited the binding of [^{32}P]pCMV-Luc to resident macrophages. The binding was also significantly inhibited by the presence of poly[I], dextran sulfate, but not by poly[C] and dextran (Fig. 3A). The inhibition profile of the cellular association at 37 °C was almost identical to that at 4 °C (Fig. 3B). EDTA did not affect the binding of [^{32}P]pCMV-Luc to the macrophages (Fig. 3A), suggesting that the plasmid binding is divalent cation-independent. Similar data were obtained in binding experiments using another plasmid, [^{32}P]pCAT (data not shown). The binding of [^{32}P]pCMV-Luc to resident macrophages was inhibited by the presence of salmon sperm DNA, likewise by the presence unlabeled pCMV-Luc in a dose-dependent manner (Fig. 4).

Cellular uptake of [Fl]pCMV-Luc was assessed by confocal laser microscopy (Fig. 5). Intracellular fluorescence was observed after incubation with [Fl]pCMV-Luc at 37 °C in a punctate pattern (Fig. 5A), indicating that the plasmid DNA was internalized via endocytosis by the resident macrophages. The uptake was abro-

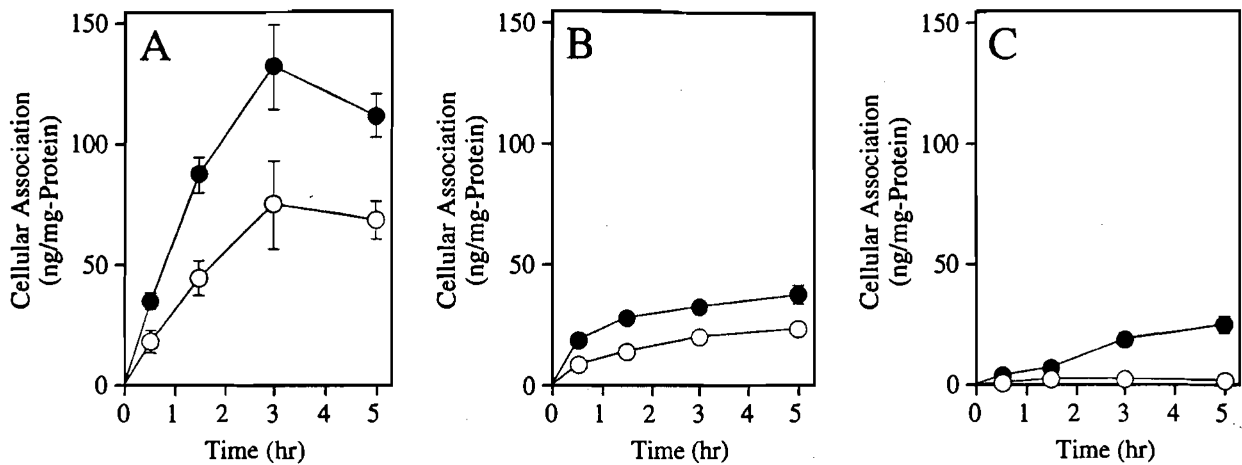

FIG. 1. Cellular association time-courses of [^{32}P]pCMV-Luc in resident macrophages (A), elicited macrophages (B), and CHO-K1 cells (C). These cells were incubated with [^{32}P]pCMV-Luc (0.1 μg/ml) at 37 °C (closed circle) or 4 °C (open circle). Each point represents the mean ± S.D. (n = 3).

gated at 4 °C and localization of the fluorescence was restricted to the cell surface (Fig. 5B). The uptake of [Fl]pCMV-Luc was inhibited by the presence of poly[I] (Fig. 5C), but not by poly[C] (Fig. 5D). These results were very consistent with those obtained with [^{32}P]-pCMV-Luc.

In the gene expression study, resident macrophages were incubated with naked pCMV-Luc at concentrations up to 100 μg/ml. However, no significant luciferase activity was obtained over this concentration range (data not shown).

DISCUSSION

To date, various types of scavenger receptor families have been identified (16-21), and the roles of these receptors have been thoroughly investigated in relation to the pathophysiology of atherosclerosis. Ligands of scavenger receptors are polyanionic molecules such as acetylated low density lipoprotein (Ac-LDL), oxidized LDL (Ox-LDL), poly[I], dextran sulfate, and bacterial lipopolysaccharide (LPS) (22). However, many polyanions including heparin, polyglutamate and poly[C] are not. Ligand binding by scavenger receptors is independent of the divalent cation (23,24). Among the scavenger receptor family, the ligand binding specificity of class AI/II scavenger receptors has been studied in detail. The extracellular collagen-like domain of the receptor is highly cationic and, thus, is a likely site of polyanionic ligand binding (25-28). The spatial distribution of the positively and negatively charged sidechains in this domain play a critical role in distinguish-

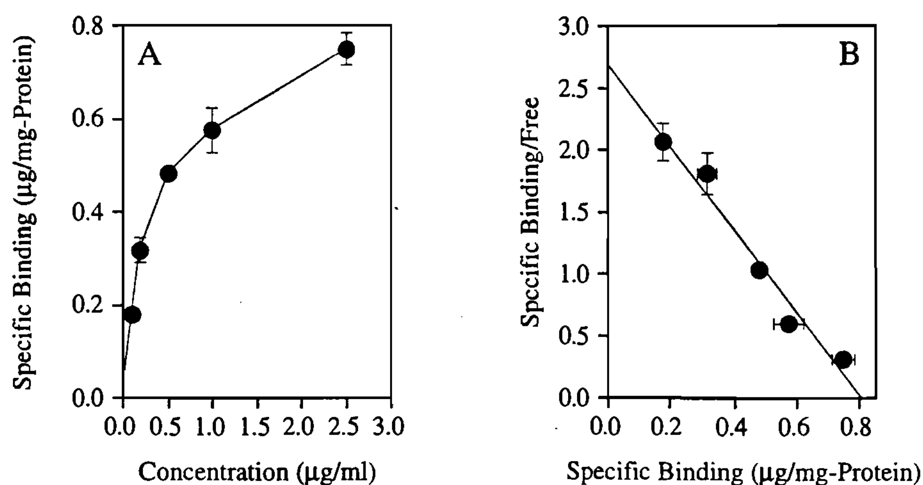

FIG. 2. Concentration-dependence of [^{32}P]pCMV-Luc binding in resident macrophages represented by a normal (A) and Scatchard (B) plot. The cells were incubated with 0.1-2.5 μg/ml [^{32}P]pCMV-Luc at 4 °C for 3 hr. Each point represents the mean ± S.D. (n = 3).

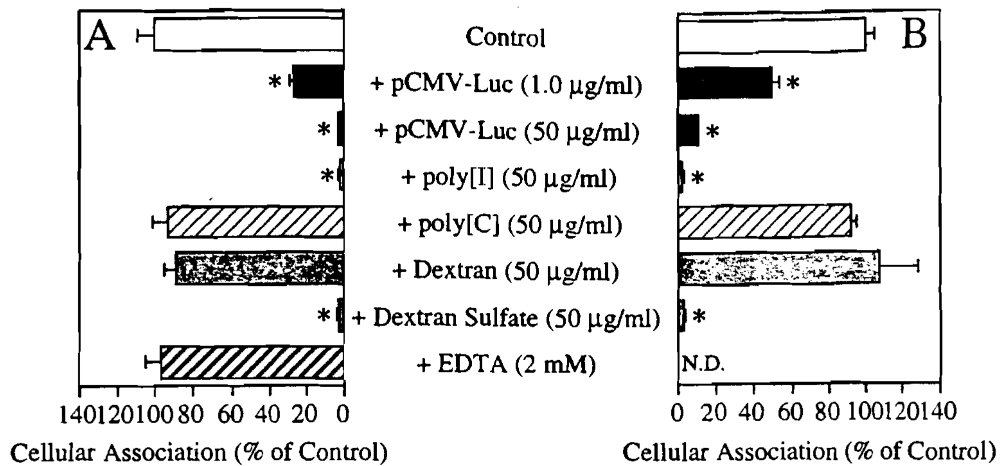

FIG. 3. Inhibition of cellular association of [^{32}P]pCMV-Luc with resident macrophages at 4 °C (A) or 37 °C (B). The cells were incubated with 0.1 μg/ml [^{32}P]pCMV-Luc for 3 hr in the presence of various inhibitors. Each point represents the mean ± S.D. (n = 3). N.D.; not determined.

ing between polyanionic ligands that bind and those that do not (12).

The present study has demonstrated that plasmid, bacterial DNA, is taken up by the resident macrophages in a specific manner for polynucleotides including salmon sperm DNA (Fig. 5). The results of competitive inhibition experiments using poly[I], poly[C] and dextran sulfate lead to the hypothesis that plasmid DNA may be recognized by the class AI/II scavenger receptor (Fig. 4). However, Pearson et al. have demonstrated that double-stranded DNA including plasmid DNA is an ineffective competitive inhibitor of the class AI scavenger receptor in CHO cells expressing the receptor (29). Oligo- and polyribonucleotides are required to form a base quartet-stabilized four stranded helix (quadruplex) to bind to class AI scavenger receptors. This conformational requirement accounts for the polyribonucleotide-binding specificity of scavenger receptors: poly[I], poly[G], and other polynucleotides are expected to exhibit stable quadruplex binding, while poly[C], a double-stranded DNA, and others that do not expected to form such quadruplexes, fail to bind. Lau et al. also reported that expression of class A scavenger

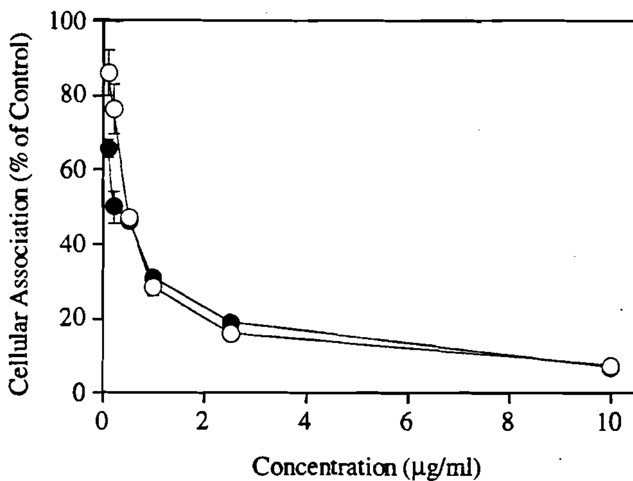

FIG. 4. Inhibition of cellular association of [^{32}P]pCMV-Luc with resident macrophages at 4 °C by salmon sperm DNA. The cells were incubated with a tracer amount of [^{32}P]pCMV-Luc for 3 hr in the presence of 0.1-10 μg/ml unlabeled pCMV-Luc (open circle) or salmon sperm DNA (closed circle). Each point represents the mean ± S.D. (n = 3).

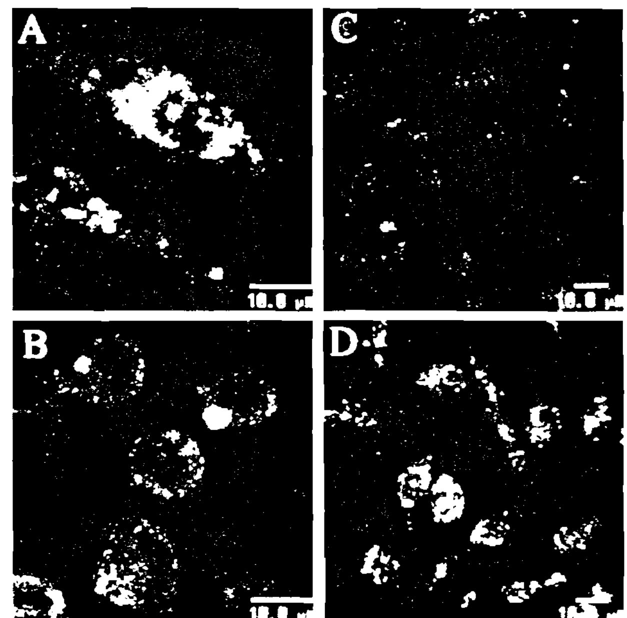

FIG. 5. Uptake of [Fl]pCMV-Luc by resident macrophages. The cells were incubated with 5.0 μg/ml [Fl]pCMV-Luc for 3 hr at 37 °C (A) or 4 °C (B). The uptake at 37 °C was inhibited by the presence of 50 μg/ml poly[I] (C), but not by 50 μg/ml poly[C] (D).

receptor in elicited macrophages was similar to that in resident macrophages (30). However, cellular association of [^{32}P]pCMV-Luc in elicited macrophages was significantly lower than that in resident macrophages (Fig. 1).

In our recent study, we found that cultured brain microvessel endothelial cells could also take up plasmid DNA in a similar manner to that observed in the peritoneal macrophages in this study (31). In the endothelial cells, low but significant luciferase activity was observed after incubation with naked plasmid DNA, which contrasts with the results of the gene expression experiments in the present study. The uptake mechanism and/or intracellular fate would differ between cell types. A high metabolic activity in macrophages might be involved in the phenomenon.

Overall, the present study has demonstrated that mouse peritoneal macrophages take up plasmid DNA by a specific mechanism mediated by a receptor like the macrophage scavenger receptor. Further studies are required to identify the receptor involved in the uptake of plasmid DNA in macrophages.

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