Characterization of Plasmid DNA Binding and Uptake by Peritoneal Macrophages from Class A Scavenger Receptor Knockout Mice

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Purpose. Plasmid DNA (pDNA) has become an important class of macromolecular agent suitable for non-viral gene therapy as well as DNA vaccination. Our recent study has suggested that pDNA is taken up by mouse peritoneal macrophages via a specific mechanism mediated by a receptor similar to the scavenger receptor (SR). This study was designed to further characterize the pDNA uptake by macrophages in order to elucidate the mechanism.

Methods. The binding and uptake of pDNA labeled with ^{32}P or a fluorescent marker were studied *in vitro* using cultured Chinese hamster ovary (CHO) cells expressing the class A scavenger receptor (SRA) and peritoneal macrophages from SRA-knockout mice.

Results. pDNA binding and uptake by CHO(SRA) cells were minimal and almost identical to that by wild-type CHO cells. Macrophages from the knockout mice showed pronounced pDNA binding and uptake as did the control macrophages. In both types of macrophage, pDNA binding was significantly inhibited by cold pDNA, polyinosinic acid and dextran sulfate but not by polycytidylic acid or Ac-LDL. These results provide direct evidence that SRA is not responsible for the significant binding and subsequent uptake of pDNA by mouse peritoneal macrophages. Further binding experiments revealed that, in addition to polyinosinic acid and dextran sulfate, heparin was a potent inhibitor among a variety of polyanionic compounds such as polynucleotides, anionic polysaccharides and modified proteins including Ox-LDL.

Conclusions. The present study suggest that pDNA binding and uptake by mouse peritoneal macrophages are mediated by a specific mechanism to some defined polyanions not by scavenger receptors. The finding would be an important basis for further studies to elucidate the mechanism(s) of pDNA uptake by macrophages.

KEY WORDS: scavenger receptors; knockout mice; peritoneal macrophages; plasmid DNA; receptor-mediated endocytosis.

INTRODUCTION

Plasmid DNA (pDNA) has become an important class of macromolecular agent suitable for non-viral gene therapy as well as DNA vaccination (1). *In vivo* application of pDNA is thought to be safer than that of viruses because there is less potential for adverse effects. Administration of large quantities, or repeated dosing, of naked pDNA can often be achieved by systemic or local injection. However, concerns have been raised (2,3) since there is increasing evidence suggesting that bacterial, but not mammalian, DNA activates immune competent cells, especially macrophages (4–7). Stacey *et al.* have demonstrated that pDNA is taken up by macrophages and activates inflammatory gene induction (4). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (5,6) and interleukin-12 (7) are produced from macrophages following stimulation with pDNA. Moreover, lethal toxic shock can be evoked by induction of macrophage-derived TNF-α after administration of bacterial DNA to mice (5,6) However, the cellular uptake mechanism of pDNA by macrophages is not yet fully understood.

Previously, we have shown that naked pDNA is rapidly taken up by the liver, preferentially by the non-parenchymal liver cells, from the bloodstream in mice (8) and we have also shown that this occurs in perfused rat liver (9). Hepatic uptake is significantly inhibited by typical ligands of the macrophage scavenger receptor (SR) such as polyinosinic acid (poly[I]), dextran sulfate and maleylated bovine serum albumin (Mal-BSA), but not by polycytidylic acid (poly[C]) (8,9). These results led us to hypothesize that the hepatic uptake of pDNA is predominantly mediated by the SR expressed on liver macrophages, Kupffer cells. The class A scavenger receptor (SRA), the best characterized SR, which recognizes a wide variety of anionic macromolecules based on their three dimensional structure (10–12), seemed a probable candidate to be responsible for pDNA uptake. However, it has been reported that double-stranded DNA including pDNA is an ineffective competitive inhibitor of the class A scavenger receptor type I in CHO cells expressing the receptor (12).

Very recently, we caried out an *in vitro* study of pDNA uptake employing cultured mouse peritoneal macrophages (13). The results we obtained suggested that pDNA is taken up by macrophages via a mechanism mediated by a receptor like the SRA. Our current study was undertaken to clarify whether pDNA uptake by macrophages is mediated by the SRA and we did this by performing a series of binding and uptake experiments using cultured CHO cells expressing SRA and peritoneal macrophages from SRA-knockout mice. The present study demonstrates that SRA is not responsible for the significant pDNA uptake by macrophages. Furthermore, the binding characteristics of pDNA to the macrophages were studied in order to have an insight into the mechanism.

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ABBREVIATIONS: pDNA, plasmid DNA; SR, scavenger receptor; SRA, class A scavenger receptor; CHO cells, Chinese hamster ovary cells, CHO(SRA) cells, CHO cells expressing SRA; poly[I], polyinosinic acid; poly[C], polycytidylic acid; poly[I:C], polyinosinic-polycytidylic acid; poly[dI:dC], polydeoxyinosinic-polydeoxycytidylic acid; Ac-LDL, acetylated low density lipoprotein; DiI-Ac-LDL, Ac-LDL labeled with 1,1′dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Ox-LDL, oxidized LDL; Suc-BSA, succinylated bovine serum albumin; Mal-BSA, maleylated bovine serum albumin.

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MATERIALS AND METHODS

Chemicals

RPMI 1640 medium and Ham's F-12 medium were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). Polyinosinic acid (poly[I],), polycytidylic acid (poly[C],), polyinosinic-polycytidylic acid (poly[I:C]), polydeoxyinosinic-polydeoxycytidylic acid (poly[dI:dC]) and dextran were purchased from Pharmacia (Uppsala, Sweden). Dextran sulfate, heparin, chondroitin sulfate-A and chondroitin sulfate-C were purchased from Nacalai Tesque (Kyoto, Japan). Dermatan sulfate and hyaluronic acid were obtained from Serbio Laboratory and Wako pure chemical industries (Kyoto, Japan), respectively. pGL3-control vector was purchased from Promega (Madison, WI). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD). Acetylated low density lipoprotein (Ac-LDL), oxidized LDL (Ox-LDL) and Ac-LDL labeled with 1,1′dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) were obtained from Biomedical Technologies, Stoughton, MA. Bovine serum albumin (BSA) derivatives, succinylated BSA (Suc-BSA) and maleylated BSA (Mal-BSA), were synthesized as described previously (9). All other chemicals used were of the highest purity available.

Preparation of pDNA

pCMV-Luc was used as a model pDNA. The pDNA was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector into the polylinker of the pcDNA3 vector. The pDNA was amplified in the DH5α strain of *Escherichia coli*, extracted and purified by a QIAGEN Plasmid Giga Kit and EndoFree Plasmid Buffer Set (QIAGEN GmbH, Hilden, Germany), and diluted with sterilized saline. Purity was confirmed by 1% of agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm. For binding and uptake experiments, pDNA was radiolabeled using [α-^{32}P]dCTP by nick translation as described by Sambrook *et al.* (14). For confocal microscopic observation, pDNA was labeled with fluorescein using a FastTag FL labeling kit (Vector Laboratories, Burlingame, CA).

CHO and CHO(SRA) Cells

Chinese hamster ovary (CHO) cells obtained from Dainippon Pharmaceutical were cultured in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml). SRA expressing the CHO cell line (CHO(SRA)) was prepared by polybrene-mediated DNA transfection (14).

Harvesting and Culture of Mouse Peritoneal Macrophages

Class A scavenger receptor type I/II (SR-AI/II) knockout mice were bred as reported previously (15). In brief, a targeting vector was introduced into exon 4 (the ligand-binding region) of the gene. Brother-sister mating of heterozygous animals was carried out to generate homozygous mutants (MSR$^{-/-}$ mice). Wild-type (MSR $^{+/+}$) mice were used as controls. Male ICR mice (20–25 g) purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) were also used for competitive binding experiments. Resident macrophages were collected from the peritoneal cavity of the 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), streptomycin (100 μg/ml) and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well for uptake experiments, or plated on 35 mm glass-bottomed microwell dishes (MatTek, Ashland, MA) for confocal microscopic observations. 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.

Cellular Binding and Uptake Studies

Binding and uptake studies were carried out using cultured CHO cells or macrophages 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]pDNA (0.1 μg/ml) was added. No degradation products were detected in the medium up to 5 hr incubation at 37°C. At the end of the incubation, the cells were washed five 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 was determined using the modified Lowry method (16) with BSA as a standard. To test the competition for binding in SRA-deficient macrophages, unlabeled polyanions such as poly[I], poly[C], dextran sulfate and Ac-LDL were added to the incubation wells together with [^{32}P]pDNA. In addition to these inhibitors, a variety of polyanions were tested in the binding experiments using the macrophages from ICR mice. The incubation was continued at 4°C for 3 hr, and the radioactivity and protein content were determined as described above.

Confocal Microscopic Study

CHO and CHO(SRA) cells were washed three times with 1.5 ml HBSS, and 100 μl HBSS containing DiI-Ac-LDL was added, with or without pDNA. After 16 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).

DiI-Ac-LDL and pDNA uptake was assessed in the macrophages. Cultured macrophages from knockout and control animals were washed three times with 1.5 ml HBSS, and 100 μg/ml HBSS containing DiI-Ac-LDL or fluorescein-labeled pDNA ([Fl]pDNA, 5.0 μg/ml) was added. After 16 hr incubation, the cells were washed, fixed with 10% neutral formalin, and subjected to confocal microscopy.

In Vivo Biodistribution Experiments

SRA-knockout and control mice were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg)

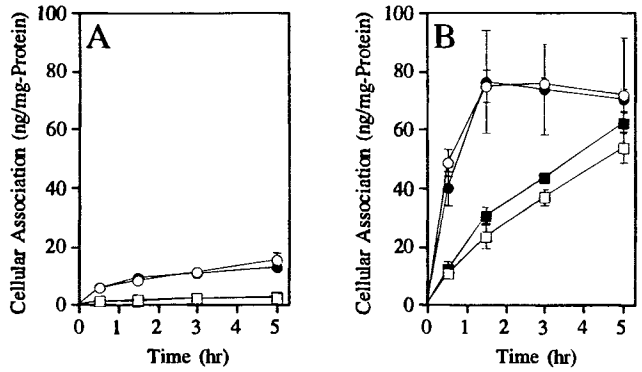

Fig. 1. Cellular association time-courses of [^{32}P]pDNA in CHO (open symbols) and CHO(SRA) (closed symbols) cells (A) and peritoneal macrophages from wild-type (open symbols) and SRA-knockout (closed symbols) mice (B). These cells were incubated with [^{32}P]pDNA (0.1 μg/ml) at 37°C (circles) or 4°C (squares). Each point represents the mean ± S.D. (n = 3). The S.D. was included in the symbol when it was very small.

and a saline solution of [^{32}P]pDNA was injected into a tail vein at a dose of 1 mg/kg. Skin incision was made to visualize a jugular vein, and blood (100 μl) was withdrawn from the vein with a heparinized 28-gauge needle syringe at 1, 3, and 5 min after injection. At 10 min, blood was collected from the vena cava after incision of the abdomen and the mice were then killed. Tissues (liver, spleen, kidney, lung, heart, and brain) were isolated, washed with saline, blotted dry, and weighed. Blood was centrifuged and plasma was separated. Plasma and a small amount of each tissue were weighed and digested with Soluene-350 during overnight incubation at 45°C. Following digestion, 0.2 ml 2 N HCl (for neutralization) and 5 ml Clear-sol I (scintillation medium) were added to each tissue and plasma sample, the samples were stored overnight, and radioactivity was measured using a scintillation counter (LSA-500, Beckman, Tokyo, Japan). Total accumulation amount in each tissue was calculated based on the weights.

RESULTS

Binding and Uptake of [^{32}P]pDNA in CHO(SRA) Cells

Figure 1A shows the time-courses of the cellular association of [^{32}P]pDNA in wild type CHO cells and CHO cells expressing SRA. Radioactivity was slowly associated with CHO(SRA) cells at 37°C in a time-dependent manner, and the association at 4°C was significantly lower. However, the profile was identical to that observed in wild-type CHO cells and significantly lower than that observed in peritoneal macrophages (Fig. 1B). The function of the expressing SRA on the CHO(SRA) cells was confirmed by the significant uptake of DiI-Ac-LDL, a typical ligand of SRA (Fig. 2B), whereas wild-type CHO cells failed to take up DiI-Ac-LDL (Fig. 2A). The uptake of DiI-Ac-LDL by CHO(SRA) cells was not affected by the presence of cold pDNA (10 μg/ml) (Fig. 2C), suggesting that pDNA is not an inhibitor of SRA expressed on the CHO cells.

Binding and Uptake of [^{32}P]pDNA and [Fl]pDNA in Macrophages from SRA-Knockout Mice

Figure 1B shows the time-courses of the cellular association of [^{32}P]pDNA with macrophages from SRA-knockout and control mice at 37°C and 4°C. A pronounced cellular association of [^{32}P]pDNA was observed in the SRA-deficient macrophages as well as the control macrophages and this was temperature-dependent.

Figure 3 illustrates the uptake of DiI-Ac-LDL and [Fl]pDNA in SRA-knockout and control macrophages assessed by confocal laser microscopy. In macrophages from SRA-knockout mice, DiI-Ac-LDL uptake was markedly reduced compared with control macrophages (Fig. 3A, B), indicating that the macrophages cannot efficiently take up the ligand due to SRA disruption (15,17,18). On the other hand, significant fluorescence derived from [Fl]pDNA was observed in both macrophages in a punctate pattern (Fig. 3C,D), suggesting that the pDNA was internalized via endocytosis. These results indicate that the SRA on the macrophages plays no significant role in pDNA uptake by the cells.

Figure 4 shows the effect of polyanionic compounds on [^{32}P]pDNA binding to control and SRA-deficient macrophages. In both macrophages, [^{32}P]pDNA binding was significantly inhibited by the presence of cold pDNA, poly[I] and dextran sulfate but not by poly[C] or Ac-LDL.

Biodistribution of [^{32}P]pDNA in Knockout Mice After Systemic Administration

In order to confirm the results of *in vitro* studies, we examined the *in vivo* pharmacokinetic characteristics of

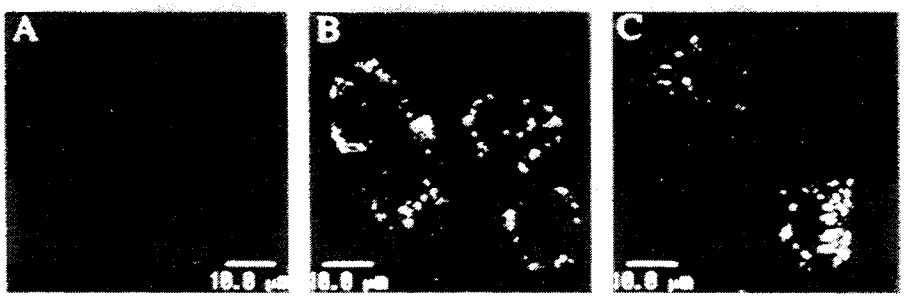

Fig. 2. Confocal microscopic images of DiI-Ac-LDL uptake by CHO cells (A) and CHO(SRA) cells (B, C). The cells were incubated with DiI-Ac-LDL for 16 hr at 37 °C in the absence (A, B) and presence (C) of 10 μg/ml pDNA. The images shown are typical among similar ones in the several visual fields.

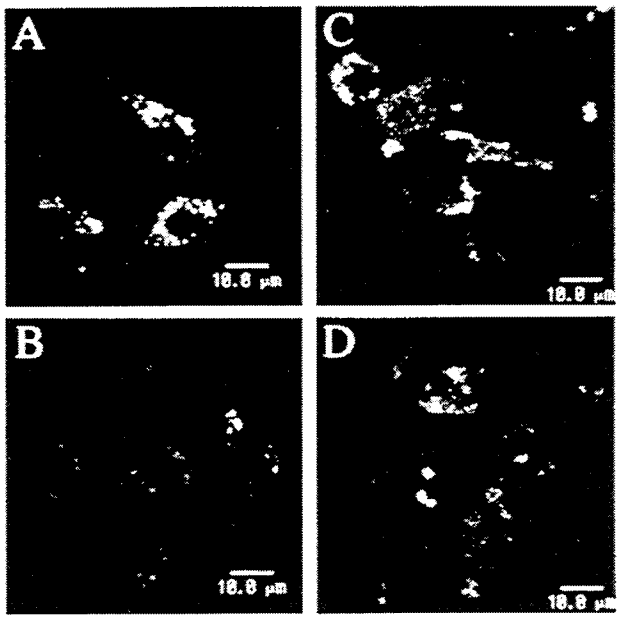

Fig. 3. Confocal microscopic images of DiI-Ac-LDL (A, B) and [Fl]pDNA (C, D) by wild-type (A, C) and SRA-knockout (B, D) macrophages. The cells were incubated with DiI-Ac-LDL or [Fl]pDNA (5.0 μg/ml) for 16 hr or 5 hr, respectively, at 37°C. The images shown are typical among similar ones in the several visual fields.

[^{32}P]pDNA in SRA-knockout and wild type mice after intravenous injection of a dose of 1 mg/kg. Radioactivity derived from [^{32}P]pDNA was eliminated from the plasma and mostly accumulated in the liver both in the knockout and control mice. Figure 5 shows the amount of [^{32}P]pDNA in the plasma, liver and other organs 10 min after administration. No significant differences were observed between the control and SRA-knockout mice as far as the amount in tissues was concerned. The plasma concentration-time curves were also similar (data not shown).

Further Characterization of [^{32}P]pDNA to Mouse Peritoneal Macrophages

In order to characterize the pDNA binding in more detail, competitive binding experiments were carried out using cultured

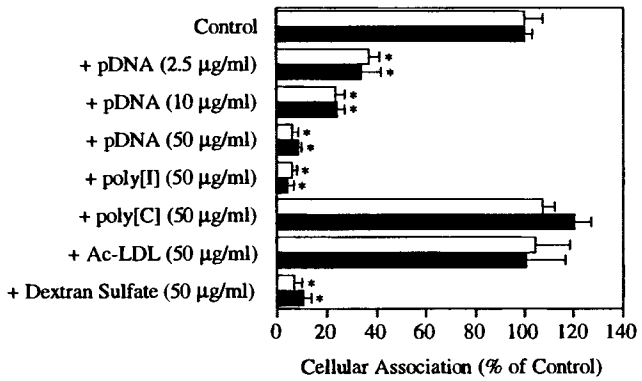

Fig. 4. Inhibition of cellular binding of [^{32}P]pDNA with wild-type (open bars) and SRA-knockout (closed bars) macrophages at 4°C. The cells were incubated with 0.1 μg/ml [^{32}P]pDNA for 3 hr in the presence of various inhibitors. Each point represents the mean ± S.D. (n = 3). *Significantly different ($p < 0.01$) from the control values.

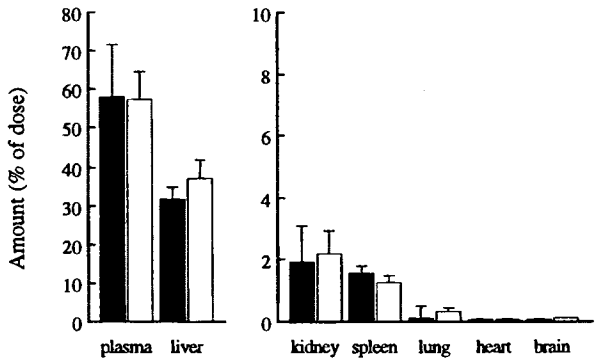

Fig. 5. Biodistribution of [^{32}P]pDNA in wild-type (open bars) and SRA-knockout (closed bars) mice 10 min after intravenous injection of a dose of 1 mg/kg.

macrophages collected from ICR mice. Figure 6 shows the effect of a variety of polyanionic compounds on [^{32}P]pDNA binding to the macrophages. Among the tested macromolecules including polynucleotides, modified proteins and polysaccharides, heparin was proved to be a potent inhibitor, in addition to poly[I] and dextran sulfate. Slight but significant inhibition was also observed in the presence of poly[dI:dC] and dermatan sulfate. Other polyanions including Ox-LDL did not affect the binding.

DISCUSSION

In order to construct the strategy for effective and safe nonviral gene therapy and DNA vaccination, it is necessary to understand the mechanism by which pDNA is taken up by macrophages, the most important cell population responsible for *in vivo* pDNA clearance and subsequent activation. The present study demonstrates that the macrophage SRA does not play an important role in the pDNA uptake by mouse peritoneal macrophages.

In our previous study, the binding of [^{32}P]pDNA to the peritoneal macrophages was insensitive to EDTA treatment (13), suggesting that, like SRA, the binding is independent of divalent cations (19). The binding characteristics are also similar to those of SRA as far as polynucleotide binding specificity (poly[I] vs. poly[C]) is concerned (Figs. 4 and 6). On the other hand, heparin, which is not a ligand of SRA (11), was proved to be a potent inhibitor of the pDNA binding (Fig. 6). In addition, Ac-LDL and Ox-LDL failed to inhibit the pDNA binding (Figs. 4 and 6). Therefore, it is unlikely that pDNA uptake by macrophages may be mediated by a receptor of the SR families on macrophages such as MARCO (20), CD36 (21) and macrosialin/CD68 (22) that have been identified as receptors for modified LDL.

In our previous report, the pDNA binding to the macrophages was saturable and 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 (13). Although further studies are required to identify the binding site, the results obtained in the present study seem to provide some new implications. It is postulated that a specific mechanism, by which a certain polyanionic nature of pDNA is recognized by the putative binding site (or receptor) on the macrophages, may be involved since some defined polyanions, such as poly[I], dextran sulfate and heparin are potent inhibitors, among various polyanionic compounds. Electrostatic interaction of some basic residues in the site with the negatively

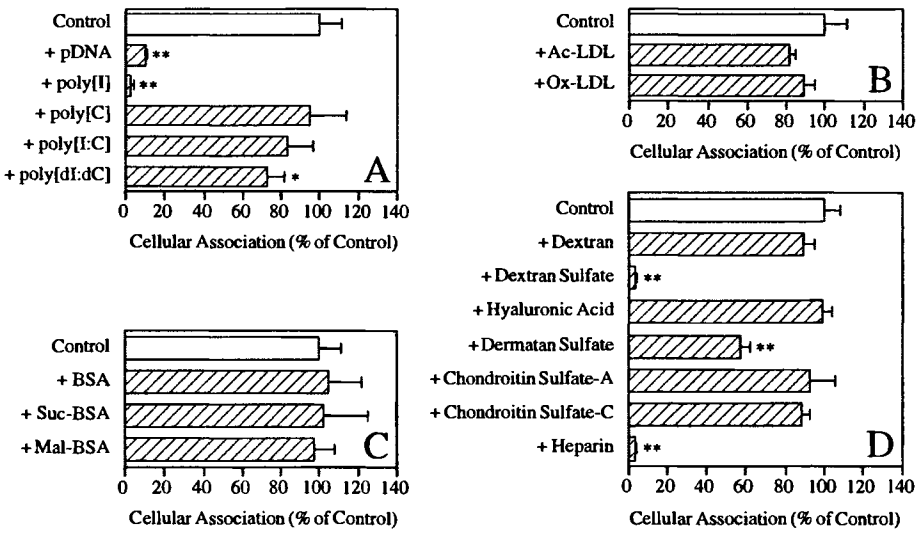

Fig. 6. Inhibition of cellular binding of [^{32}P]pDNA with macrophages from ICR mice by nucleic acids (A), modified LDL (B), modified BSA (C) and polysaccharides (D) at 4°C. The cells were incubated with 0.1 μg/ml [^{32}P]pDNA for 3 hr in the presence of various inhibitors (50 μg/ml). Each point represents the mean ± S.D. (n = 3). *, ** Significantly different ($p < 0.05$ and 0.01, respectively) from the control values.

charged phosphate groups of pDNA might account for the results. A high density of negative charges of macromolecules might be important for the binding. Poly [G], poly[I] and other particular polynucleotides are known to form a base-quartet-stabilized for-strand helix (quadruplex) (11,12). Presumably, this hyperstructure would be highly polyanionic. Similar speculation could be applied to polysaccharides. A synthetic polysaccharide, dextran sulfate, and heparin are usually more highly sulfated, on a basis of a disaccharide unit, than dermatan sulfate and chondroitin sulfate, ineffective glycosaminoglycans. Hyaluronic acid, which is nonsulfated glycosaminoglycan with less negative charges, is also ineffective.

In efforts to elucidate the pathogenesis of systemic lupus erythematosus, in which anti-DNA antibodies are postulated to be involved, DNA binding proteins or receptors have been identified in various cells including human leukocytes (23). Several DNA binding proteins that bind to lower molecular weight DNA, oligodeoxynucleotide as antisense molecules, have also been discovered in a variety of cell types. Recently Mac-1 (CD11b/CD18), a leukocyte integrin involved in leukocyte adhesion and migration, has been show to be a functional oligonucleotide receptor on human polymorphonuclear leukocytes (24). It should be noted that heparin, a potent inhibitor of pDNA binding to the macrophages, is a typical ligand of Mac-1 (25). Moreover, the profile observed in the competitive binding experiments using glycosaminoglycans in macrophages (Fig. 6D) is similar to the ligand specificity of Mac-1, i.e., heparin and dermatan sulfate interact with Mac-1 while chondroitin sulfates and hyaluronic acid do not (26). On the other hand, it is reported that Mac-1 expression on elicited macrophages is higher than that on resident macrophages (27). This is in contrast to our previous finding that pDNA uptake is significantly reduced in elicited macrophages compared with resident macrophages (13). Although we cannot exclude the possibility that Mac-1 might be involved in pDNA uptake by macrophages, further experiments are required to clarify the point.

Emlen *et al.* have demonstrated in a series of mouse liver perfusion experiments that single-stranded DNA is bound to liver sinusoidal cells, primarily Kupffer cells (28). Although the hepatic binding of DNA has been suggested to be mediated by a receptor, binding properties have not been studied in detail. Our previous studies also demonstrated that [^{32}P]pDNA is efficiently eliminated from the bloodstream by preferential uptake by liver nonparenchymal cells probably by Kupffer cells, liver resident macrophages, as well as degradation by nucleases (8). Although *in vitro* experiments using peritoneal macrophages showed SRA is not responsible for pDNA uptake, we carried out *in vivo* experiments to confirm the finding. In the present study, biodistribution of [^{32}P]pDNA was examined up to 10 min after injection in order to minimize the effect of pDNA breakdown by nucleases. The hepatic uptake of [^{32}P]pDNA in SRA-knockout mice was similar to that in control mice (Fig. 5), indicating that SRA on Kupffer cells played no significant role in the elimination of pDNA from the circulation.

In conclusion, the present study has provided direct evidence that SRA is not responsible for pDNA uptake by mouse peritoneal macrophages. This study also offers novel implications in relation to the pDNA binding characteristics to the macrophages. These findings would be an important basis for further studies to elucidate the mechanism(s) of pDNA uptake by macrophages.

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