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Uptake and Gene Expression of Naked Plasmid DNA in Cultured Brain Microvessel Endothelial Cells

Masaru Nakamura, Pablo Davila-Zavala, Hideaki Tokuda, Yoshinobu Takakura, and Mitsuru Hashida[1]

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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Cellular uptake and gene expression of plasmid DNA and its cationic liposome complexes were studied using primary cultures of bovine brain microvessel endothelial cells (BMEC) developed as an in vitro model of the blood-brain barrier. An avid association of naked plasmid DNA with the BMEC monolayer was observed at 37°C, which is comparable to that of the DNA/liposome complex.The cellular association significantly decreased at low temperature (4°C). The binding at 4°C was saturable and significantly inhibited by polyanions involving polyinosinic acid and dextran sulfate, typical ligands for the macrophage scavenger receptors, but not by polycytidylic acid or in the presence of EDTA. Unexpectedly, a significant gene expression in the BMEC was obtained by transfection with naked plasmid DNA although the expression level was lower than that obtained by plasmid DNA/cationic liposome complex. Taken together, cultured capillary endothelial cells derived from the brain are able to take up naked plasmid DNA via a scavenger receptor like-mediated mechanism for polyanions and gene expression in the cells takes place.

The vascular capillary endothelial cells are an attractive target for gene transfer because of their large population with a huge surface area and contiguity with the bloodstream (1-3). Their location at the parenchymal-blood interface is ideal for delivering transgene products either systemically or locally. Although endothelial cells in any tissues can be the target for gene delivery, brain capillary endothelium comprising the blood-brain barrier (BBB), which restricts the passage of most water soluble molecules including proteins from the cerebrovasucular circulation to the brain, will be of particular interest (4-6). Brain microvessel endothelial cells, when transfected with the gene encoding a secretory therapeutic protein, may be utilized as a delivery vehicle that secretes the gene product into the brain. This strategy could be an alternative way to the intravascular delivery of therapeutic proteins to the brain across the BBB (7).

The purpose of this study was to elucidate the cellular interaction and gene expression of a non-viral vector system, plasmid DNA complexed with cationic liposome, in primary cultures of bovine brain endothelial cells (BMEC), developed as an in vitro model of the BBB (4-6). Here we report that the BMEC can take up naked plasmid DNA via a scavenger receptor-mediated mechanism specific for polyanions and a significant gene expression can be obtained without any transfection regents.

MATERIALS AND METHODS

Chemicals. Polyinosinic acid (poly [I], average M.W. 103.3 kDa), polycytidylic acid (poly [C], average M.W. 99.5 kDa) were purchased from Pharmacia Biotech (Tokyo, Japan). Dextran sulfate (DS-150, average M.W. 150 kDa) was purchased from Nacalai Tesque (Kyoto, Japan). Dextran sulfate (DS-8, M.W. 8 kDa) was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). [α-^{32}P]-dCTP (3000 Ci/mmol) was obtained from Amersham (Tokyo, Japan). pCMV-CAT and pGL3-Control Vector were purchased from Promega (Madison, WI, U.S.A.). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Lipofectin was purchased from GIBCO BRL (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

Construction and 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

[1] To whom correspondence should be addressed: Mitsuru Hashida, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan. Fax: 81-75-753-4575. E-mail: hashidam@pharm.kyoto-u.ac.jp.

Abbreviations: BBB, blood-brain barrier; BMEC, brain microvessel endothelial cells; HUVEC, human umbilical vein endothelial cells; pCMV-CAT, plasmid DNA encoding chloramphenicol acetyltransferase under the control of cytomegalovirus promoter; pCMV-Luc, plasmid DNA encoding firefly luciferase under the control of cytomegalovirus promoter; Poly-I, polyinosinic acid; Poly-C, polycytidylic acid; DS, dextran sulfate.

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vector. Plasmid DNA was amplified in E. coli strain DH5α and then isolated and purified by QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). 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. The plasmid was labeled with [α-^{32}P]-dCTP by nick translation (8).

Cell culture. Bovine brain microvessel endothelial cells (BMEC) were isolated from the cerebral gray matter of bovine brains as described previously (4,5). Isolated BMEC were suspended in culture medium (MEM/F-12 with 10 % horse serum), seeded in 6 or 24-well culture plates pretreated with rat tail collagen and fibronectin at a density of approximately 5×10^4 cells/cm^2 and grown to half-confluence (5 days-old in culture) or confluence (9-11 days-old in culture). The in vitro model system has been shown to possess all features of the BBB including tight intercellular junctions, the lack of membrane fenestrations, γ-glutamyl transpeptidase and alkaline phosphatase activities, factor VIII antigen, and amino acid and hexose transport systems (9-12).

The cell line of human umbilical vein endothelial cells (HUVEC, ECV 304) was purchased from ATCC, maintained in Medium 199 with 10% FBS, seeded in 6 or 24-well culture plates at a density of approximately 4×10^3 cells/cm^2 and grown to confluence (6 days-old in culture).

Cellular association study in BMEC and HUVEC. Cellular association studies were carried out using confluent cell monolayers of BMEC or HUVEC in 24-well culture plates. Cells were washed three times and incubated with HBSS for 20 min at 37°C or 4°C. After the preincubation, HBSS was removed and 0.5 ml of HBSS containing 0.1 μg/ml of 32P-labeled plasmid DNA (pCMV-CAT) or its cationic liposome complexes (DNA : Lipofectin; 0.1 : 0.5 μg/ml) was added. In the binding experiment at 4°C, non specific binding was defined as binding in the presence of 100 μg/ml of unlabeled plasmid DNA and subtracted from the total binding to calculate the specific binding. The non-specific binding was approximately 40 % of the total binding (non-specific binding plus specific binding). The cell monolayers were washed five times with 0.5 ml of ice-cold HBSS at appropriate time intervals and then solubilized with 1.0 ml of 0.3 M NaOH with 0.1 % Triton X-100 for 1 hr at 37°C. Aliquots were taken for determination of radioactivity and protein content. The radioactivity of 32P was measured witha liquid scintillation system (LSC-500, Beckman, Tokyo, Japan) after the addition of 5 ml of scintillation medium (Cleasol I, Nacalai Tesque, Kyoto, Japan) to each sample. The Protein content was determined by modified Lowry method (13).

Luciferase gene transfection study in BMEC and HUVEC. Transfection efficiencies of naked pCMV-Luc and pCMV-Luc-cationic liposome complexes were examined using cultured BMEC of 5 or 10 days-old cells or HUVEC. The cells in one well of 6-well plate were transfected with various amounts of naked plasmid DNA and its cationic liposome complexes. The cells were first rinsed twice with PBS to remove serum, and serum-free Opti-MEM I medium (1 ml) containing naked plasmid DNA or its cationic liposome complexes was added to each well. The transfection solution was removed after 6 h and replaced with fresh MEM/Ham's F-12 medium containing 10 % horse serum for BMEC and Medium 199 for HUVEC. Two days after transfection, the cells were washed with PBS (Ca and Mg free) and harvested by scraping and transferred to Eppendorf tubes.

For luciferase assay, the cells were subjected to three cycles of freezing and thawing (liquid N2 for 3 min, 3 min at 37°C) and centrifuged at 14,000 rpm for 3 min, and the supernatants were stored at −20dgC until the luciferase assays were performed. Ten microliterof the supernatant was mixed in 100 μl of luciferase assay buffer and the light units produced immediately was measured on a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as relative light units per mg protein.

RESULTS AND DISCUSSION

In a previous study, we found that succinylated and maleylated bovine serum albumins were taken up by BMEC in a specific manner for polyanions (14). These findings prompted us to examine the cellular interaction and subsequent gene expression of polyanionic plasmid DNA in BMEC together with those of DNA/cationic liposome complex.

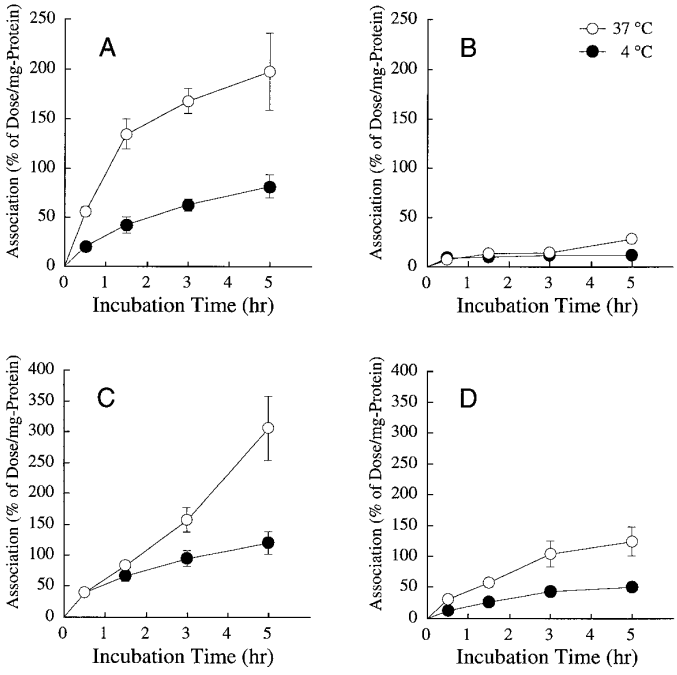

FIG. 1. Cellular association time courses of ^{32}P-labeled naked plasmid DNA and its cationic liposome complexes in BMEC and HUVEC. Confluent BMEC or HUVEC were incubated with ^{32}P-labeled naked plasmid DNA (pCMV-CAT, 0.1 μg/ml) (A and B) or Lipofectin complex (pCMV-CAT : Lipofectin, 0.1 : 0.5 μg/ml) (C and D) at 37°C or 4°C. Each point represents the mean ± S.D. values (n=3).

Fig. 1 shows the cellular association time courses of ^{32}P-labeled naked plasmid DNA (pCMV-CAT) and its cationic liposome complex in BMEC and HUVEC at 37 and 4°C. Naked plasmid DNA was associated with the BMEC monolayers to a great extent in a temperature-dependent manner (Fig. 1A), suggesting that this highly negative polynucleotide can bind to the cell surface of BMEC and subsequently be taken up by the cells. Meanwhile, much less association of naked plasmid DNA was observed for HUVEC and it was almost independent of temperature (Fig. 1B). These results imply that HUVEC may do not have specific uptake mechanism for naked plasmid DNA. Cationic liposome complex showed different association profiles (Fig. 1C; 1D) In BMEC, the apparent association amount of cationic liposome complexes increased linearly up to 5 hr at 37°C although there was no large difference in the association amounts between naked DNA and its cationic liposome complex. In HUVEC, the association was significantly increased and temperature-dependent.

The cellular association time course study demonstrates that BMEC have an uptake mechanism for na-

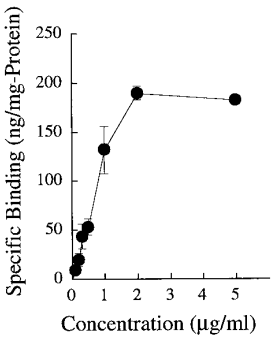

FIG. 2. Concentration-dependent binding of ^{32}P-labeled naked plasmid DNA to BMEC. Confluent BMEC were incubated with various concentrations of ^{32}P-labeled naked plasmid DNA (pCMV-CAT) for 3 hours at 4°C. Each point represents the mean ± S.D. values (n=2).

ked plasmid DNA. Therefore, we further studied the binding characteristic of naked plasmid DNA to BMEC. Fig. 2 shows the concentration dependent binding of ^{32}P-labeled naked plasmid DNA (pCMV-CAT) to BMEC. The binding was saturable in the concentration range up to 5 μg/ml. Dissociation constant (Kd) and maximal binding amount (Bmax) were estimated to be about 2.0 (μg/ml) and 0.3 (μg/mg-Protein), respectively. The Bmax value corresponds to approximately 6×10^4 binding sites per cell.

To further explore the binding characteristics of plasmid DNA in BMEC, we investigated the effect of polyanions on the binding of naked plasmid DNA (pCMV-CAT) to BMEC at 4°C. The binding of plasmid DNA was significantly inhibited by DS-150 (Fig. 3B) and Poly-I (Fig. 3C), typical ligands for the macrophage scavenger receptor (15,16), but not by Poly-C (Fig. 3D) which is not recognized by the receptor (15,16). The 50% inhibitory concentrations (IC_{50}) of Poly-I and DS-150 were 2.0 and 0.35 μg/ml, respectively. DS-8 was a less potent inhibitor for the binding (Fig. 3A) and no significant inhibition was observed in the tested range. Moreover, the binding of naked plasmid DNA to BMEC was not affected in the presence of EDTA (2.5 mM) (data not shown), indicating that the binding is independent of divalent cations. This phenomenon was also characteristic to the macrophage scavenger receptor (15,17). Taken together, these results suggested that plasmid DNA is taken up by BMEC via a specific mechanism mediated by (a) receptor(s) like the macrophage scavenger receptor. Similar results were obtained for plasmid DNA uptake in cultured mouse peritoneal macrophages (unpublished data).

Our previous study has demonstrated that the BMEC can take up polyanions such as acetylated low-density lipoprotein (Ac-LDL), dextran sulfate and large succinylated proteins (14). De Vries et al (18) have characterized Ac-LDL binding and uptake in primary cultures of bovine BMEC and reported that Kd and Bmax of Ac-LDL were 5.4 (μg/ml) and 0.286 to 0.626 (μg/mg-Protein), respectively. Very recently, the expression of the class A type I and type II macrophage scavenger receptors in isolated bovine brain microvessel has been confirmed by a reverse transcriptase PCR study (19). These findings suggest that the cultured BMEC used in this study may express the macrophage scavenger receptors that might be responsible for plasmid DNA uptake. However, plasmid DNA reportedly is not a ligand for the macrophage scavenger receptor. Pearson et al. has shown that, in contrast to polynucleotides which form four-stranded helices, double stranded DNA including a plasmid are ineffective inhibitors in Ac-LDL degradation assay using CHO cells expressing bovine type I scavenger receptors (20). The authors conclude that the spacial distribution of the negatively charged phosphates in polynucleotide quadruplexes may form a charged surface which is complementary to the positively charged surface of the collagenous ligand-binding domain of the scavenger receptor. Other scavenger receptors such as oxidized LDL receptor recently cloned from bovine endothelial cells (21) might be involved in the uptake of plasmid DNA by the cultured BMEC. In any cases, three-dimensional structure of plasmid DNA would be important in recognition of the receptor on BMEC. Further studies are required to elucidate the uptake mechanism.

Based on the uptake and binding studies, transfection experiments were carried out. We employed 5 and 10 days-old BMEC as the proliferating cells and quies-

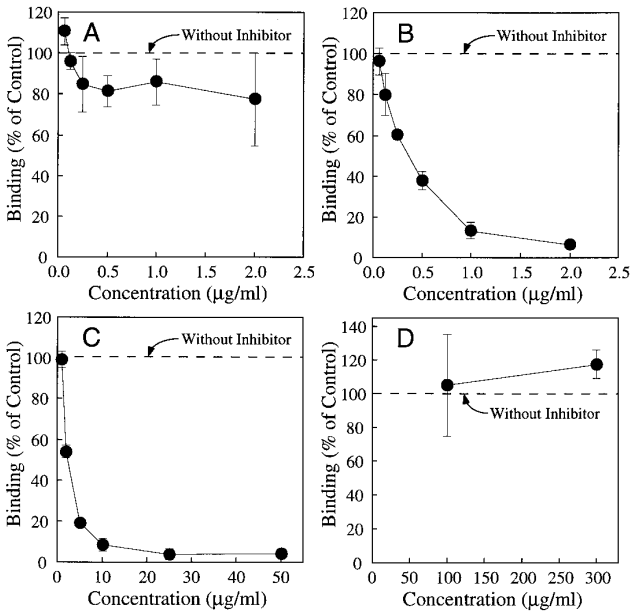

FIG. 3. Effect of polyanions on the binding of naked plasmid DNA to BMEC. Confluent BMEC were incubated with ^{32}P-labeled naked plasmid DNA (pCMV-CAT, 0.1 μg/ml) together with various concentrations of polyanions for 3 hours at 4°C. Each point represents the mean ± S.D. values (n=4).

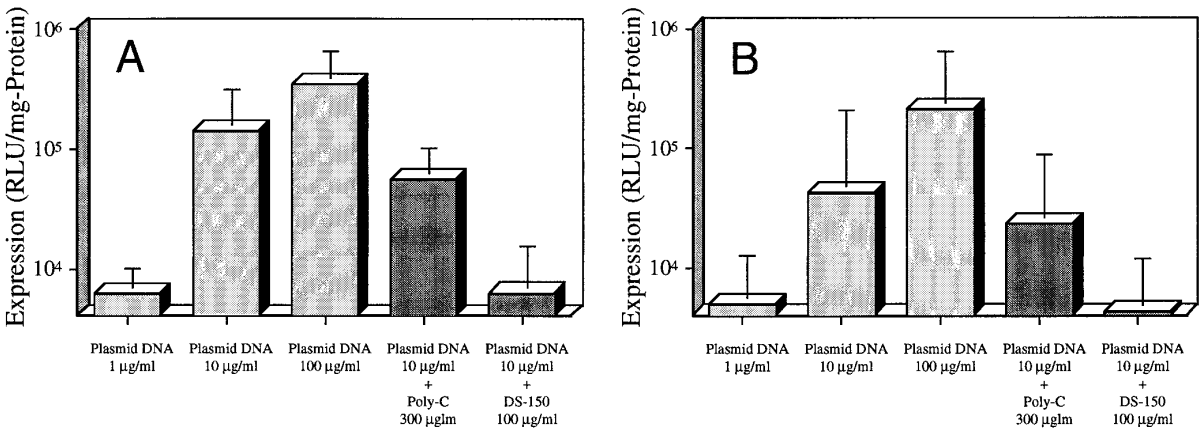

FIG. 4. Dose-dependent gene expression and effect of polyanions on the expression in BMEC. BMEC (A; 5 days-old in culture, B; 10 days-old in culture) were transfected with various concentrations of naked plasmid DNA (pCMV-Luc). At the dose of 10 μg/ml of naked plasmid DNA, gene expression was also examined in the presence of Poly-C (300 μg/ml) or DS-150 (100 μg/ml).

cent cells forming a confluent mololayer, respectively. Prior to gene transfer with DNA/cationic liposome complexes, we performed transfection experiments without the transfection reagent since naked plasmid DNA was thought to be taken up by the cells. We found that a significant gene expression could be obtained by plasmid DNA alone and the level of gene expression depended on the dose of plasmid in both BMEC (Fig. 4A and B). In addition, the gene expression was inhibited in the presence of DS-150, but not of poly-C.

Fig. 5 shows the luciferase activities in BMEC and HUVEC after transfection with naked plasmid DNA (pCMV-Luc) and its cationic liposome complex. The cationic liposome complex successfully transfected the BMEC with culture age of 5 and 10 days (Fig. 5A and B) and HUVEC (Fig. 5C). The gene expression level with naked DNA was lower than that obtained by its cationic liposome complex. Interestingly, the luciferase activities in HUVEC after transfection with DNA alone were significantly lower than those in BMEC. This result was consistent with the association amounts of naked plasmid DNA in these endothelial cells (Fig. 1). Furthermore, the growing BMEC at an early age (day 5, Fig. 5A) after transfection with the liposome complex expressed the luciferase gene much more efficiently than the older confluent cells (day 10, Fig. 5B). It is generally known that rapidly growing cells are susceptible to transfection (22). A mitotic event is required for efficient transfection since the nuclear membrane disintegrates during mitosis, which would facilitate the delivery of DNA to the nuclear compartment. On the other hand, culture age of BMEC did not markedly affect the gene expression level in the case of transfection with DNA alone. The mechanism underlying the gene expression after naked DNA transfection in the BMEC remains to be elucidated.

Thus, this study demonstrated that microvessel endothelial cells obtained from the brain have the ability to take up naked plasmid DNA via a scavenger receptor-mediated mechanism. Gene expression in

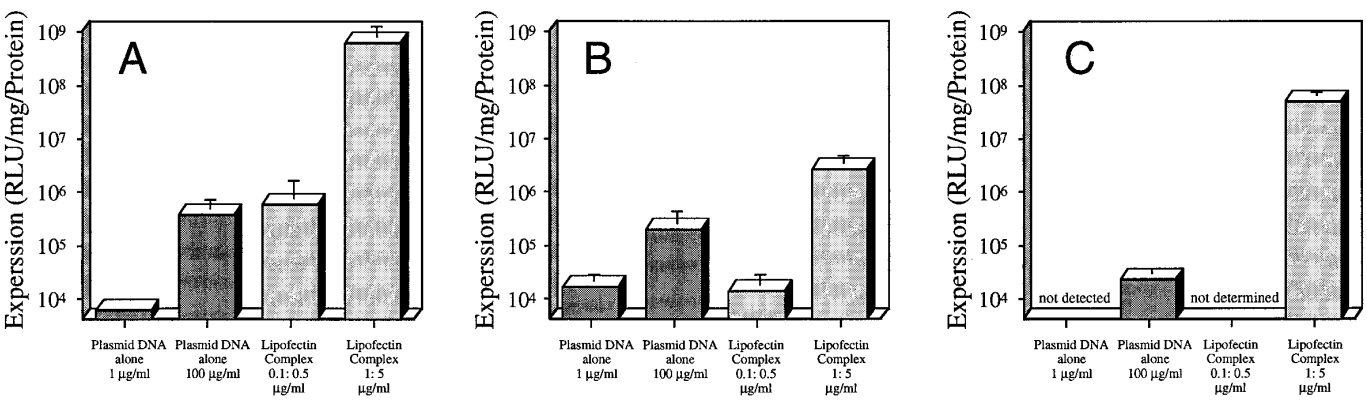

FIG. 5. Effect of lipofectin on gene expression in BMEC and comparison with HUVEC. BMEC (A; 5 days-old in culture, B; 10 days-old in culture) and HUVEC (C) were transfected with naked plasmid DNA (pCMV-Luc) or its lipofectin complex.

BMEC occurs without any transfection agents. However, the transfection efficacy by naked DNA seems not to be satisfactory. Cationic liposome or other appropriate vector systems should be utilized for efficient gene transfer to the brain microvessel endothelial cells aiming at therapeutic protein delivery to the brain. Otherwise, the findings on naked plasmid DNA in the present study may provide useful information for non-viral gene therapy since naked plasmid DNA has become an important class in somatic gene therapy (23,24). One may need to consider the possibility of unexpected gene expression in the cerebral endothelial cells in the case of gene therapy using a large amount of naked DNA (23).

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