

Impact of Convective Flow on the Cellular Uptake and Transfection Activity of Lipoplex and Adenovirus

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An *in vitro* cell culture model that mimics *in vivo* extracellular environment would be useful in developing *in vivo* gene delivery system. In the present study, a parallel flow model was applied to investigate the impact of convective flow on cellular uptake and transfection activity in endothelial cells. LipofectAMINE PLUS and adenovirus were used as model vectors, which bind cells *via* electrostatic- and ligand-receptor interactions, respectively. Whereas a convective flow increased the total amount of vector passing through the flow chamber by 3 orders of magnitude, uptake was increased by less than 10-fold, suggesting that the flow severely inhibited cellular uptake by reducing the retention time in the chamber and/or by diminishing the affinity between the cell and vector. Moreover, the uptake of both vectors was increased in a shear stress-dependent manner to a comparable extent, suggesting that the effect of flow on the cellular uptake was not significant. In contrast, transfection efficiency (TE), expressed as the transfection activity normalized by the cellular uptake of vectors was dramatically stimulated by shear stress, only when LipofectAMINE PLUS was used. Since the activities of the CMV promoter were unaffected by a shear stress, it is possible that altered intracellular trafficking may responsible for the improvement in lipoplex-mediated TE, presumably related to the cellular uptake pathway.

Key words convective flow; gene delivery; adenovirus; lipoplex

The targeting of therapeutic genes to various organs *via* systemic administration is an ideal concept for non-invasive gene therapy in curing malignant diseases. To realize a sophisticated gene delivery system, efforts have been made to develop viral and non-viral vectors. As a result, certain types of transfection tools such as lipoplexes, polyplexes and adenoviruses are currently available, which can exhibit a high transfection activity for *in vitro* cultured cells. However, even when non-viral vectors and adenovirus vectors are administered to the systemic circulation *in vivo*, the transfection activities are exclusively limited to the lung^{1–4}) and liver,^{5,6}) respectively. In the case of lipoplexes and polyplexes, they immediately form large aggregates with erythrocytes,^{1–4}) and then rapidly accumulate in the lung.^{7,8}) Similarly, the transgene expression of adenovirus is exclusively limited to the liver after an i.v. administration of adenovirus mainly due to the high hepatic clearance^{5,9,10}) *via* multiple binding between hepatic cells and adenovirus^{5,6}) (*e.g.* CAR-fiber, integrin-RGD motif and fiber shaft-heparan sulfate interactions).

However, considering that blood flow rate in an artery (5.6 l/min in human¹¹) and *ca.* 460 ml/min in guinea pig¹²) and blood volume (*ca.* 5.4 l in 70 kg human body weight¹³) and *ca.* 200 ml in 2.3 kg guinea pig body weight¹²), gene vectors are calculated to be pumped out and back to the heart within approximately 1 min. In other words, gene vectors are able to pass through various organs many times before their elimination from the systemic circulation. This is inconsistent with the fact that transgene expression is barely detectable in organs such as the heart, kidney and muscle except for the lung or liver.

The discrepancy between *in vivo* and *in vitro* transfection activity can be attributed to differences in the extracellular environments of the target cells. One factor that has been clearly identified is plasma protein. When vectors are admin-

istered to the systemic circulation, many types of plasma proteins, including lipoproteins, bind to the vectors, resulting in a drastic loss in transfection efficiency.^{14,15}) On the other hand, in a typical *in vitro* transfection, vectors are applied using serum-free culture medium. Another factor that may affect the *in vivo* transfection activity is blood flow. Since current *in vitro* transfection studies have been carried out under static condition, it is difficult to estimate the effect of fluid flow on transfection activity. Two possible effects are possible; namely, physical effect and biological effect. In the former case, convective flow would wash out the vectors before they reach the cellular surface by sedimentation, and /or it may affect an intrinsic affinity constant for the association between vector and cell. In the latter case, certain types of mechano-sensors (*e.g.* receptor tyrosine kinases¹⁶), signaling pathways (*e.g.* protein kinase C¹⁷) and the expression of endogenous genes (*e.g.* platelet-derived growth factor^{18,19}) could affect a response to shear stress, which is generally loaded at a border of cellular surface and convective flow in horizontal direction.²⁰) Since transfection activity is highly dependent on intracellular trafficking and intranuclear transcription,^{21–23}) shear stress may change the transfection efficiency of transgenes that are taken up by cells.

In the present study, we investigate the influence of convective flow on cellular uptake and transfection activity by means of a parallel flow model. For a comparison, lipoplex and adenovirus vectors which are taken up *via* absorptive- and receptor-mediated endocytosis, respectively, were used in an attempt to clarify the uptake mechanism-dependent influence of flow on transfection activity from physical and biological points of view.

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MATERIALS AND METHODS

Materials MBEC4 cells derived from mouse brain endothelial cells were generously supplied by Dr. T. Tsuruo and Dr. M. Naito (Tokyo University, Japan). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 0.5 $\mu\text{g}/\text{ml}$ heparin sulfate under an atmosphere of 5% CO_2/air at 37 °C. To prepare the reporter gene vector for the pDNA (pcDNA3.1-GL3), an insert fragment encoding luciferase (GL3) was obtained by HindIII/XbaI digestion of the pGL3-basic vector (Promega, Madison, WI, U.S.A.), and ligated to the HindIII/XbaI digested site of the pcDNA3.1 (Invitrogen Corp, Carlsbad, CA, U.S.A.). LipofectAMINE PLUS was obtained from the Invitrogen Corp. All other chemicals used were commercially available and were reagent grade. Concerning the viral vector, the E_1^- , E_3^- , a replication-deficient serotype 5 adenovirus in which an expression cassette was inserted in the E_1 position was used.²⁴⁾ In addition, an RGD peptide was inserted in the HI loop of the fiber knob to improve transfection activity to the mouse-derived cells.²⁵⁾ The expression cassette consisted of a cytomegalovirus promoter/enhancer, cDNA encoding luciferase (GL3) and BGH polyadenylation sequences which were also encoded in the pDNA used in the LipofectAMINE PLUS-mediated transfection.

Parallel Flow Device A parallel flow model was established, as described in a previous study with minor modifications.^{26,27)} A rectangular channel ($14 \times 4 \text{ mm}^2$) was compartmentalized by silicon films (0.3 mm height) on the slide slips (Matsunami, Osaka, Japan), and 2×10^4 cells were then seeded on the channel after coating with laminin, and the cells were then cultured for 40 h until reaching confluence. Slides were mounted on the chamber as illustrated in Fig. 1. The flow loop, reservoirs and infusion pump (Pharmacia, LKB·Pump P500) were perfused with Krebs-Ringer bicarbonate buffer (4.8 mM KCl, 1.0 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.5 mM CaCl_2 , 120 mM NaCl, 24 mM NaHCO_3 , 12.5 mM HEPES, 5.0 mM Glucose: pH 7.4) containing 8.0 μg DNA/30 ml of lipoplex or 1.0×10^9 particle titer/30 ml of adenovirus. The upstream flow loop was then connected to the inlet port to allow the perfusate flow in the chamber. The fluid flow from the chamber outlet port was recovered in the reservoir and the medium was recirculated throughout the trans-

fection experiments. To maintain the temperature of the perfusate passing through the flow chamber at 37 °C, the upstream flow loop was temporarily warmed up by water bath at 41–42 °C. The shear stresses (τ) were adjusted to 2.3 and 9.7 dyn/cm^2 by controlling the flow rate (Q) so as to mimic the characteristics of *in vivo* cardiovascular and venous, respectively²⁷⁾ based on the following equation:

$$\tau = \frac{6Q\mu}{bh^2}$$

where μ , b and h denote the viscosity of the fluid perfusate, channel width (0.4 cm) and channel height (0.03 cm), respectively. The flow chamber positioned vertically during the perfusion.

As a control, cells were incubated with 16.8 μl of Krebs-Ringer buffer containing adenovirus and LipofectAMINE PLUS under static conditions, in which the concentration of vectors and fluid volume in the chamber was adjusted to that used in the perfusion study. To avoid a drying, cells were incubated in humid box. In the perfusion experiment, 8.7 and 35.6 μg of pDNA, and 1.1×10^9 and 4.5×10^9 particles of adenovirus was passed through the parallel flow chamber at 2.3 dyn/cm^2 and 9.7 dyn/cm^2 , respectively. As a static control, we adjusted the concentration of the vector to the flow conditions (4.9 ng of pDNA and 5.6×10^5 particle titer of adenovirus in 16.8 μl Krebs-Ringer buffer) since adjustment of the total dose to the flow conditions led to a highly toxic effect.

Quantification of Cellular Uptake of Exogenous DNA pDNA was quantified as demonstrated previously by means of real-time PCR.²³⁾ After incubation with LipofectAMINE PLUS and adenovirus for 30 min, the cells were treated with trypsin to detach them from the slide slip. For quantification of the cellular luciferase genes, plasmid DNA and adenovirus genome DNA was purified from cell lysates by means of a GenElute Mammalian Genome DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, U.S.A.), and subjected to TaqMan PCR with an ABI PRISM® 7700 Sequence Detection System. The sequence of the probe was 5'-CCGCTGAATTGGAATCCATCTTGCTC-3' with FAM as a fluorescent dye on the 5' end and TAMRA as a fluorescence quencher dye labeled to the 3' end. This probe is designed to anneal to the target between the sense primer (5'-TTGACCGCCT-

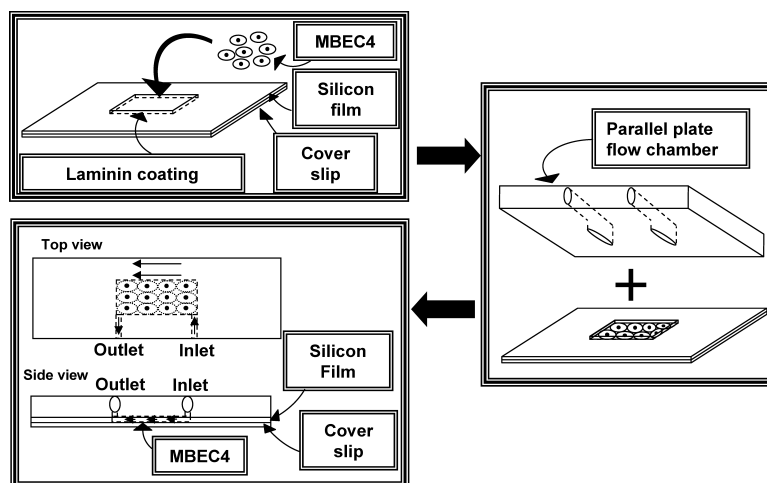


Fig. 1. Schematic Diagram Illustrating the Parallel Flow Model

GAAGTCTCTGA-3') and the antisense primer (5'-ACAC-CTGCGTCGAAGATGTTG-3') in the luciferase sequence. As a reference, a dilution series of pDNA3.1-GL3 was run along with the virus sample.

Transfection Activity After perfusion of the lipoplex and adenovirus for 30 min, the slide slip was transferred to the cell culture dish, and incubated in the culture medium for a further 6 h. The cells were then washed, and lysed with reporter lysis buffer (Promega, Madison, WI, U.S.A.). Luciferase activity was initiated by the addition of 50 μ l of luciferase assay reagent (Promega) to 20 μ l of cell lysate, and measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysates was determined using a BCA protein assay kit (PIERCE, Rockford, IL, U.S.A.).

To establish stably transfected MBEC4 cells expressing CMV-driven luciferase, luciferase (GL3)-encoding pcDNA3.1 was transfected into MBEC4 cells by LipofectAMINE PLUS and selected by treatment with 800 μ g/ml of G418 for two weeks. The luciferase-positive cell population was maintained in the growth media with 400 μ g/ml of G418.

RESULTS AND DISCUSSION

The effect of a convective flow on transfection activity was compared between adenovirus and lipoplex from both physical and biological points of view. Concerning the physical effect, the cellular uptake of lipoplex and adenovirus vector was compared between static and flow conditions. Since physicochemical characters such as size and density are distinct from each other, the velocity of sedimentation (V_{sed}) should be also different following Stokes' equation. When a perfusion study is carried out using horizontally seeded cells, cellular uptake may be also affected by the amount of vector, which can sediment on the cell surface during convection through the chamber.²⁷⁾ To avoid the effect of different sedimentation efficiencies, cell cultures were positioned vertically in the perfusion study.

After perfusion for 30 min, cells were collected to quantify cellular uptake by means of real-time PCR (Fig. 2). Cellular uptake is represented as the percent of the control. As a result, the cellular uptake of adenovirus was increased by 1.2-fold and 3.5-fold by convective flow at 2.3 dyn/cm² and 9.7 dyn/cm², respectively, compared with static conditions (Fig. 2A). Similarly, the uptake of LipofectAMINE PLUS was also increased by 1.9-fold and 5.8-fold, respectively (Fig. 2B). Cellular uptake was enhanced by stimulating the cellular association of the vector due to fluid motion and by the increased amount of dose passing through the flow chamber. Furthermore, cellular uptake was enhanced to the same extent (approximately 3-folds) when the flow rate was increased from 2.3 to 9.7 dyn/cm² for both vectors, indicating that the impact of flow on cellular uptake between LipofectAMINE PLUS and adenovirus was not significant. The enhancement in cellular uptake (less than 10 fold) was much less prominent than that for the dose passing through the chamber (1935-fold and 7946-fold higher than static conditions at 2.3 dyn/cm² and 9.7 dyn/cm², respectively). This suggests that the collision of vectors with the cellular surface is severely perturbed by convective flow since unbound vectors gaining access to the cellular surface were washed out and/or

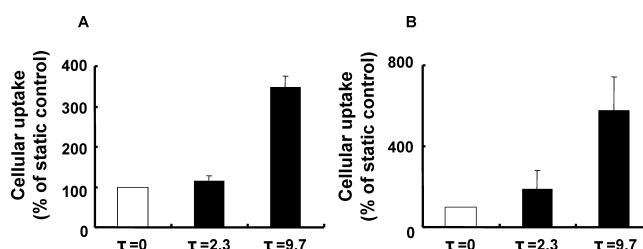


Fig. 2. Effect of Convective Flow on the Cellular Uptake of Adenovirus (A) and LipofectAMINE PLUS (B)

Adenovirus (A) and LipofectAMINE PLUS (B) was perfused with Krebs-Ringer bicarbonate containing 8.0 μ g DNA/30 ml of lipoplex or 1.0×10^9 particle titer/30 ml of adenovirus for 30 min. As a control, cells were incubated with 16.8 μ l of Krebs-Ringer buffer containing adenovirus and LipofectAMINE PLUS under static conditions, in which the concentration of vector and fluid volumes in the chamber were adjusted to the perfusion study. Cellular uptake was quantified by means of TaqMan PCR. Cellular uptake is represented as percent of static condition. Each bar represent the mean and standard deviation of three experiments.

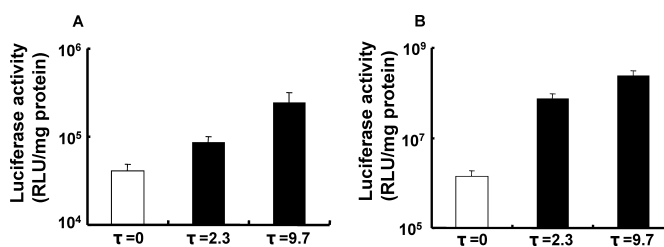


Fig. 3. Effect of Convective Flow on the Transfection Activity of Adenovirus (A) and LipofectAMINE PLUS (B)

Cells were incubated with adenovirus (A) and LipofectAMINE PLUS (B) under static and shear stress conditions for 30 min. Cells were further incubated in cell culture medium for an additional 6 h. Transgene expression was evaluated by luciferase activity. Each bar represent the mean and standard deviation of three experiments.

the affinity between vector and cell surface was decreased.

As shown in Fig. 2B, the cellular uptake of the lipoplex increased monotonically depending on the shear stress. This is inconsistent with a previous observation reporting that cellular uptake was temporarily increased at 2.3 dyn/cm² and then decreased when the stress was further increased to 9.7 dyn/cm².²⁷⁾ The overall influence of shear stress on the cellular uptake is balanced by a stimulative effect which induces collisions between vectors and cells, and by a negative effect which prevents the association and/or enhances the dissociation between cells and vectors²⁸⁾ by its hydrodynamic force. This discrepancy can be accounted for by assuming that the cellular binding of LipofectAMINE PLUS is stronger than that of TransIT (Panvela) which was used in the previous study.²⁷⁾

The biological effect of shear stress was investigated by measuring transfection activity. In adenovirus, transgene expression was stimulated by a convective flow by 2.1 times and 5.9 times at 2.3 dyn/cm² and 9.7 dyn/cm², respectively compared with static conditions (Fig. 3A). Transfection efficiency (TE) calculated as transfection activity normalized by the cellular uptake of adenovirus is summarized in Table 1. TE values are quite comparable values regardless of the extent of shear stress. This indicates that the transfection activity is directly related to the cellular uptake of adenoviral DNA under the flow conditions. In contrast, the transgene expression of LipofectAMINE PLUS was drastically increased by 53 times at 2.3 dyn/cm² (Fig. 3B), whereas cellular uptake was increased by only 1.9 times (Fig. 2B). As a result, the TE

Table 1. Cellular Uptake, Transfection Activity, and Transfection Efficiency (TE) (% of Static Control)

	Cellular uptake (A) (% of static control)	Transfection activity (B) (% of static control)	TE (B/A)
Adenovirus ($\tau=2.3$)	116 \pm 12	212 \pm 31	1.83
Adenovirus ($\tau=9.7$)	349 \pm 27	588 \pm 180	1.68
LipofectAMINE PLUS ($\tau=2.3$)	190 \pm 90	5330 \pm 1590	28.1
LipofectAMINE PLUS ($\tau=9.7$)	576 \pm 166	17800 \pm 4630	30.9

The cellular uptake is measured by real-time PCR. The transfection activity is measured by luciferase assay and protein assay. TE is calculated by dividing B by A.

value at 2.3 dyn/cm² was calculated to be 28 times higher than that under static conditions, as summarized in the Table 1. This suggests that TE of pDNA taken up by the cells were stimulated by a shear stress. In contrast to the differences between static conditions and low shear stress conditions (2.3 dyn/cm²), the TE values were quite comparable, regardless of the extent of shear stress (2.3 dyn/cm² and 9.7 dyn/cm²). Therefore, a low shear stress was sufficient to trigger a certain types of intracellular mechanism related to increasing TE.

It is possible that the drastic improvement in TE by shear stress in Lipoplex-mediated transfection is due to the stimulation of the activity of the CMV promoter, since previous reports showed that expression level of endogenous genes was altered under conditions of shear stress.¹⁹⁾ The intrinsic activities of endogenous transcription factors associated with the CMV promoter were compared between static and shear stress conditions by measuring the luciferase activity of stable transfectants, expressing CMV-driven luciferase-encoding pDNA (pDNA3.1-Luc). As a result, the stable transfectant exhibited a comparable luciferase activity (Fig. 4), suggesting that the intrinsic activity of transcription factors is not affected by shear stress. Intracellular trafficking is more susceptible to be responsible for the improvement in TE compared with intranuclear transcription process.

The mechanism underlying the lipoplex-specific increase in TE is illustrated in Fig. 5. Previous studies showed that shear stress induced the production of intracellular signaling-related proteins such protein kinase C,¹⁷⁾ MAP kinase²⁹⁾ and Rho GTPases,³⁰⁾ which control the various clathrin-independent pathways such as pinocytosis³¹⁾ and phagocytosis.³²⁾ In the case of adenovirus, binding is based on specific ligand-receptor interactions between the fiber proteins and coxsackievirus and the adenovirus receptor (CAR), and between the RGD motif in the penton base and integrin.³³⁾ Thus, it is plausible that the cellular uptake pathway of adenovirus is limited to the receptor-mediated endocytosis. In contrast, plasmid DNA condensed with LipofectAMINE PLUS is able to bind to the entire cell surface area *via* electrostatic interactions. If novel pathway was stimulated by shear stress, lipoplex could then internalize *via* alternative pathways, in addition to classical endocytosis. Currently, we have clarified that transfection activity is closely related to the cellular uptake pathway, which determines intracellular fate. It should be noted that gene vector taken up *via* macropinocytosis can avoid the lysosomal degradation, and resulted in the high transfection activity.³⁴⁾ Therefore, it is possible that shear stress may alter the cellular uptake route of a lipoplex, which is advantageous for the transgene-expression.

Collectively, impact of convective flow on cellular uptake

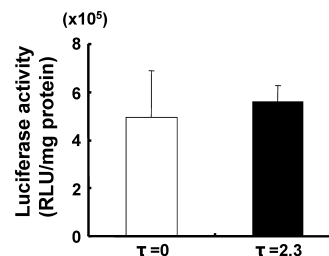


Fig. 4. Effect of Convective Flow on the Intrinsic Activity of Transcription Factors for the CMV Promoter

MBEC4 cells stably expressing luciferase were seeded on the parallel flow chamber. Luciferase activity was measured before and after the exposure to convective flow at 2.3 dyn/cm² for 30 min. Each bar represents the mean and standard deviation of three experiments.

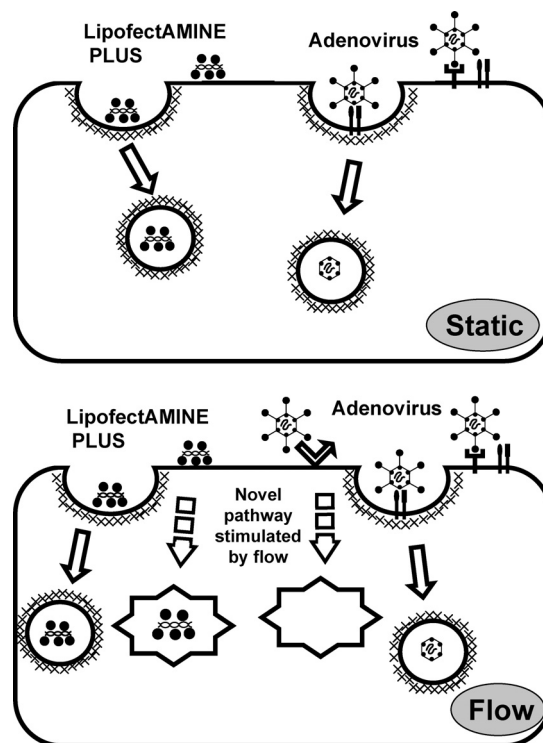


Fig. 5. Schematic Diagram Illustrating the Possible Cellular Uptake Pathways of Adenovirus and LipofectAMINE PLUS Under the Static and Flow Condition

and transfection activity was investigated with LipofectAMINE PLUS and adenovirus as models, which interact with cells *via* electrostatic and ligand-receptor interactions, respectively. Regarding the cellular uptake process, convective flow severely perturbed the cellular uptake of both vectors to the same extent by washing out the vector from the chamber before they reached cellular surface, and/or reducing the

affinity between the vector and the cellular surface. In contrast, transfection efficiency, denoted as transfection activity normalized by the cellular uptake of exogenous DNA, was drastically stimulated by shear stress only in the case of LipofectAMINE PLUS. Considering that the intrinsic activities of endogenous transcription factors associated with the CMV promoter were unaffected by shear stress, it is possible that intracellular trafficking, presumably linked to the cellular uptake mechanism, may be responsible for the improved lipoplex-mediated transfection.

This is the first demonstration, in which the effects of convective flow on the cellular uptake and transfection activity have been assessed separately. Further studies with other cell lines derived from endothelial cells (*e.g.* HUVEC) are valuable to lead a generality of this phenomenon. This model system will be useful for developing a flow-resistance to gene delivery system.

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