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HIGH-EFFICIENCY TRANSFORMATION OF HUMAN ENDOTHELIAL CELLS BY APO E-MEDIATED TRANSFECTION WITH PLASMID DNA

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Endothelial cells, because of their proximity to the blood stream, provide an attractive system for gene transfer and delivery of gene products that control foci of vascular disease processes. We describe a simple, new methodology to achieve highly efficient transformation of cultured human endothelial cells derived from umbilical veins (HUVEC). A plasmid pCH110 containing coding region for β-galactosidase driven by SV 40 early promoter region was employed to transfect HUVEC.The developed protocol exploits the role of apolipoprotein E(Apo E)in the metabolism of Apo E-containing lipoproteins and its high affinity binding to LDL receptors. DNA transfection of cultured HUVEC was carried out using standard transfection methods including calcium phosphate precipitation, polybrene mediated transfection, and lipofection. The new methodology of transfecting HUVEC employed Apo E adsorbed lipofection reagent-DNA complex, and was found to be the most efficient procedure to transform HUVEC in comparison to the standard methods used in this study.

Vascular endothelium, a confluent monolayer of flattened rhomboid-shaped endothelial cells (EC), is situated at the vital interface between the circulating blood and the surrounding tissue. Due to their location at the blood-surface interface, EC have been identified as an excellent vehicle for gene transfer and delivery of gene products. In order to understand and to engineer EC gene expression, transfection of EC with a marker gene has been reported (1-6). The ideal, *in vivo*, recipient site for EC that have undergone *ex vivo* or *in vivo* gene transfer has not been identified yet, however, transduced EC have been seeded onto synthetic vascular grafts. These transduced EC seeded grafts were implanted into the canine carotid artery from which the cells were harvested, and expression of inserted lacZ gene was documented (3). The feasibility of in vivo transplantation of transduced EC into arterial blood vessels (2), capillary bed of skeletal muscle (5), and of transfecting directly the EC of the arterial wall (6) has been explored.

The expression of recombinant genes *in vivo* utilizing EC represents a novel approach, but vascular cell gene transfer technology is consistently compromised by: (i) inefficiency of transduction, (ii) reintroduction of small number of transduced cells into the vessel walls, and (iii)

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failure to achieve a meaningful level of gene expression by directly transfecting EC of the arterial wall. The technology of seeding EC that have undergone *ex vivo* gene transfer onto prosthetic materials such as vascular grafts may offer particular advantages, but EC show little or no adhesion and proliferation on these materials.

We have developed a new methodology that provides highly efficient transfection of human endothelial cells in culture. The transfection protocol employed apolipoprotein E (Apo E) adsorption onto liposomes to mediate binding of the lipofection reagent-DNA complex to EC. Apo E is a protein molecule found on very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and high density lipoprotein (HDL), and is believed to mediate binding of Apo E containing lipoproteins with high affinity to the remnant or LDL receptor located on the cells (7). It was hypothesized that Apo E would facilitate membrane fusion of the liposomes containing DNA to the EC membrane thereby significantly increasing transfection efficiency. In this study, human umbilical vein endothelial cells (HUVEC) were transfected with a plasmid pCH110 using standard transfection methods including calcium phosphate precipitation, polybrene mediated transfection and lipofection. This plasmid contains the lacZ marker gene which codes for the bacterial enzyme β-galactosidase, driven by the simian virus 40 (SV 40) early promoter region, and allowed us to identify successfully transfected HUVEC by a histochemical method using X-gal. Thus, the transfection obtained employing the above standard technique was compared to Apo E mediated DNA delivery. We found a significant increase in HUVEC transfection using Apo E mediated lipofection compared to the standard methods.

MATERIALS AND METHODS

Medium M-199, penicillin, streptomycin, amphotericin B, water-soluble heparin, Hanks' calcium-magnesium-free phosphate-buffered saline (HBSS), Dulbeco's calcium-magnesium-free phosphate buffered saline (PBS-CMF), Lipofection Reagent, EDTA, and trypsin were obtained from Gibco BRL, Burlington, Ontario, Canada. Endothelial cell growth supplement (ECGS) came from Collaborative Biomed. Products, Bedford, Massachusetts, USA. Defined fetal bovine serum (FBS) was purchased from Hyclone Laboratory Inc., Utah, USA. CLS-II collagenase was purchased from Washington Biochemical, Freehold, New Jersey, USA Polybrene, and human collagen were obtained from Sigma Chemical, Saint Louis, Missouri, USA. Dil-Ac-LDL was purchased from Biomedical Technology Inc., Stoughton, Massachusetts, USA. 5-bromo-4-chloro-3-β-D galactopyranoside (X-Gal) was purchased from Boehringer Mannheim, Montreal, Quebec, Canada. Other Chemical used were from Fisher Chemical, Ltd, Montreal, Quebec, Canada.

HUVEC Culture: The procedure used for Harvesting of human endothelial cells from umbilical veins was essentially identical to previously published literature (8), with a few minor changes (9). HUVEC in cultures were confirmed by their characteristic polygonal shape and "cobble stone" growth pattern and by the presence of receptor for acetylated form of LDL labelled with fluorescent probe (Dil-acetyl LDL). HUVEC were cultured on collagen coated 35 mm cultured dishes in the presence of complete growth medium. The cultures were incubated at 37°C in 5% CO_2 / 95% air in a humidified incubator. The complete growth medium was obtained by supplementing M-199 medium with 20% FBS, 25 μg/ml amphotericin B, 100 μg/ml streptomycin and penicillin, 30 μg/ml

heparin, and 60 μg/ml ECGS. The growth medium was changed every 48 hours. HUVEC at the third passage were employed in transfection studies.

Transfection of HUVEC: Twenty-four hours before transfecting HUVEC, the cells were harvested during exponential growth by trypsinization and replated at a cell density of $1x10^6$ cells/35 mm culture dishes and cultured in complete growth medium. Apo E was purified from VLDL according to a published procedure (10). When the HUVEC monolayer reached 75-85% confluence, HUVEC were then transfected with calcium phosphate (11), and polybrene (12) transfection protocols. Transfection experiments were performed in triplicate. Lipofection mediated, and Apo mediated lipofection transfection protocols are as follow.

Lipofection: The pCH110 plasmid and liposome, 1:1 (wt/wt) formulation of the cationic lipid (Lipofection Reagent, Gibco BRL) were diluted separately in 100 μl of serum free medium. Three different ratio of DNA to lipofection reagent (wt/wt) were tested. The ratio of DNA to liposomes was 1:3, 1;5, and 1:8. The quantity of DNA used for lipofection was 5 μg/35 mm culture dish. The separately diluted lipofection reagent and pCH110 solutions were combined in a polystyrene tube and incubated at room temperature for 15 minutes. pCH110-lipofection reagent complex was overlaid onto the cells. Immediately following, 800 μl of serum-free medium was added to each 35 mm culture dish. Cultures were incubated for 6 or 10 hours, and the 1 ml of complete growth medium was added to each dish. After the lipofection, cells were cultured for additional period of 48 hours with complete growth medium before assaying for transient expression of β-galactosidae activity.

Apo E Mediated Lipofection: The pCH110 plasmid and liposome, a 1:1 (wt/wt) formulation of the cationic lipid (Lipofection Reagent) were diluted separately in 100 μl of serum free medium. The ratio of DNA to lipofection was 1:5. The amount of DNA used for Apo E mediated lipofection was 5 μg/35 mm culture dish. The separately diluted lipofection reagent and pCH110 were combined in a polystyrene tube and incubated for 15 minutes. Following the incubation period, 500 μl of Apo E was added to the tube and incubated at room temperature for additional 15 minutes to allow the Apo E to adsorb onto liposome. The resulting 700 μl of Apo E-adsorbed-pCH110 plasmid-lipofection reagent was overlaid onto HUVEC. Immediately following , 800 μl of serum-free medium was added to each culture dish. After 10 hours of incubation, 1 ml of complete growth medium was added to each dish. Assay for the transient expression of β-galactosidae activity was carried out after the cells have been cultured for additional 48 hours.

The presence of β-galactosidase gene product determined by staining HUVEC with X-Gal (13). Transduced HUVEC with β-galactosidase gene exhibited blue-green cytoplasm staining with X-Gal.

RESULTS AND DISCUSSION

In order to determine the number of transduced cells expressing lacZ, blue-green staining cells were counted on 6 areas on each 35 mm culture dish using an inverted transmitted-light microscope (Labovert, Ernst Leitz Wetzlar GmbH Germany) at 100x maginification. As a negative control, we used the same transfection procedure but without DNA plasmid. In such set-ups, no blue-green cytoplasmic staining of cells was observed in any of negative control experiments. The percentage of HUVEC demonstrating successful transfection with plasmid pCH110 varied according to time and method of transfection employed. These results are presented in Table I and Table II.

The transfection of HUVEC using calcium mediated precipitation caused enormous cytotoxicity resulting in extensive cell lysis. The transfection rate of the relatively few cells that remained viable was negligible. The harsh conditions (osmatic gradients) associated with this method of transfection (*ex vivo*) coupled to the limited availability of human endothelial cells from

TABLE I*. Efficiency of various methods used to transfect cultured human umbilical vein endothelial cells (HUVEC) with plasmid DNA pCH110

Methods	DNA Concentration μg	DNA:Liposomes Ratio	Transduced HUVEC %
Calcium Phosphate	5	----	Negligible
Polybrene*	5	----	3.00 ± 0.32
Lipofection-1*	5	1:3	5.00 ± 0.50
Lipofection-2*	5	1:5	6.50 ± 0.21
Lipofection-3*	5	1:8	6.37 ± 1.48

***Transfection time 6 hours.**

a tissue such as autologus saphenous vein in a clinical setting makes this procedure inadequate. The transfection efficiency of HUVEC using polybrene mediated transfection was found to be 3%.

The transfection of HUVEC using the lipofection method was carried out with a different ratio of DNA to liposomes while maintaining the concentration of plasmid pCH110 at 5 μg/culture dish. This resulted in five different lipofection experiments, and were labelled as Lipofection-1 through Lipofection-5 (Table I and Table II). In lipofection-1 to Lipofection-3 the concentration of liposome increased from 15 μg to 40 μg per culture dish. No cytotoxicity was observed when Lipofection-1, and Lipofection-2 were employed, whereas with Lipofection-3 some cell death was noticed. When the transfection time was set for six hours, Lipofection-3 yielded the higher transfection efficiency (Table I). HUVEC transfection with Lipofection-2 was found to be statistically greater when compared with that obtained with Lipofection-1 ($p < 0.01$) (Table III).

TABLE II. Effect of adsorbed ApoE onto DNA-liposomes complex and of Transfection time on the transfection efficiency (Transfection time 10 hours)

Methods	DNA Concentration μg	DNA:Liposomes Ratio	Transduced HUVEC %
Lipofection-4	5	1:3	14.90 ± 1.10
Lipofection-5	5	1:5	16.60 ± 1.30
ApoE-Lipofection*	5	1:5	22.62 ± 1.20

***ApoE concentration 500 μl.**

TABLE III. Comparison of various methods used to transfect HUVEC

Methods		p value <
Lipofection-1[#]	Polybrene	0.01
Lipofection-2[#]	Lipofection-1[#]	0.01
Lipofection-5[*]	Lipofection-2[#]	0.001
Lipofectio-5[*]	Lipofection-4[*]	0.20
ApoE-Lipofection[*]	Lipofection-5[*]	0.01

[#]Transfection time 6 hours.
[*]Transfection time 10 hours.

In the case of Lipofection-4 and Lipofection-5, the transfection time was increased to ten hours. When the results obtained with Lipofection-5 were compared with that of Lipofection-2, a significant increase in the transfection efficiency was observed ($p < 0.001$). We compared Lipofection-5 to Apo E mediated lipofection at ten hours of transfection time (Table III), ApoE mediated lipofection efficiency to transfecting HUVEC was significantly higher than Lipofection-5 with a p value less that 0.01.

In this study, we addressed the issue of low transfection efficiency associated with procedures for transfecting EC. We varied two parameters in the lipofection procedures, namely (1) the ratio of plasmid DNA to liposome, and (2) the transfection time.

We found that the highest level of HUVEC transfection was attained using Lipofection-5 when the ratio of plasmid DNA to liposome was 1:5 and the transfection time was 10 hours. Apo E is one of many protein constituents found on lipoprotein molecules i. e. VLDL, IDL, and HDL. One of the major roles of Apo E in the metabolism of lipoproteins is its ability to mediate high affinity binding to LDL receptors. The LDL receptor is located on EC. The affinity with which the Apo E protein recognise and binds to its LDL receptor, would mediate the binding and cellular uptake of the Apo E containing liposomes leading to increased transfection efficiency of EC. A comparison between the transfection of EC utilizing Lipofection-5 versus Apo E mediated transfection demonstrate a significantly higher transfection efficiency using Apo-E mediated lipofection (Table III). The methodology developed in this study takes advantage of the simple carrier system of lipofection and the high affinity binding of Apo E to the EC membrane. This technique may provide a useful tool

in the study of the molecular, and clinical aspects of transduced EC alone or in conjunction with surface modified prosthetic biomaterials on which EC monlayer can be established within clinically relevent times (14).

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