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Transfection of Small Numbers of Human Endothelial Cells by Electroporation and Synthetic Amphiphiles

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Objectives: *this study compared the efficiency of electroporation and synthetic amphiphiles (SAINT-2^{pp}/DOPE) in transfecting small numbers of human endothelial cells.*
Methods and results: *optimal transfection conditions were tested and appeared to be 400 V and 960 mF for electroporation and a 10:1 ratio for concentrations of SAINT-2^{pp}/DOPE: plasmid. Using these conditions, cell concentrations were lowered step-wise and we were able to transfect as few as one thousand cells with both methods. For detection of transfection of a small number of cells a sensitive assay was needed (Luciferase). A plasmid containing the neomycin resistance gene was used to determine the transfection rate expressed in colony forming units by counting colonies after selection. At low plasmid concentrations this transfection rate was within the same range for both electroporation and SAINT-2^{pp}/DOPE transfection. Fluorescent* in situ *hybridisation of metaphase chromosomes of transfected endothelial cells using the plasmid as a probe showed that stable integration was possible with both methods.*
Conclusions: *electroporation and a synthetic amphiphile, SAINT-2^{pp}, provide the possibility of transfecting small numbers of cells resulting in stable integration of low plasmid concentrations. The availability of this technology is important in order to obtain functional endothelial cell lines from various human blood vessels for research purposes.*

Key Words: Electroporation; Endothelial cells; Lipofection; Synthetic amphiphile; Thrombosis; Transfection.

Introduction

Various vascular disorders, such as deep vein thrombosis, myocardial infarction and graft occlusion, are related to injury or functional abnormalities of endothelial cells (EC). In order to investigate the role of EC it may be valuable to culture EC from blood vessels in which these disorders actually occur. However, these vessels cannot easily be obtained and only a small amount of material will be available. Furthermore, cultures of human EC have a limited life span and lose their original functions during culture, so it would be useful to produce cell lines with an infinite life span which have preserved their original haemostatic functions. In this study we used human umbilical vein endothelial cells (HUVEC) as a model to transfect effectively a minimal number of EC.

Commonly used non-infectious methods for transfection of mammalian cells are calcium phosphate precipitation, DEAE-dextran mediated transfection, lipofection and electroporation. For EC, calcium phosphate and DEAE-dextran are less effective.[1-7] As for lipofection of EC, varying results have been reported, depending on the cationic lipid used.[1-5,7,8] Successful attempts have been made to improve these methods by using strontium phosphate instead of calcium phosphate,[3] or Apo E-mediated lipofection.[5] Nevertheless, electroporation is still the most efficient transfection method for EC, in spite of the low cell viability.[3,4,7-12]

SAINT-2^{pp} is a novel synthetic amphiphile, the synthesis of which was recently reported.[13] It appeared to be less toxic than other amphiphiles used for lipofection.[14] Moreover, this compound, mixed with DOPE (1:1), transfects cells with an efficiency of an order of magnitude better than that obtained for lipofection, which has been most commonly used for transfection thus far. In this study we compared SAINT-2^{pp}/DOPE mediated lipofection with electroporation in their efficiency to transfect small numbers of HUVEC.

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Materials and Methods

Materials

Chymotrypsin and gelatin were obtained from Sigma Chemical Co (U.S.A.). Tryptin/EDTA, RPMI 1640 and L-glutamine came from Gibco (Scotland). Heparin was used from Leo Pharmaceutical Products B.V. (The Netherlands). Streptomycin was obtained from Radiumfarma-Fisiopharma (Italy) and penicillin from Yamanouchi Pharma B.V. (The Netherlands). Tissue culture flasks (25, 75 and 165 cm^2), 100 mm cell culture dishes, 6-well clusters and cell scrapers were purchased from Costar (U.S.A.).

Reporter plasmids (pCAT control vector and pCMVLuc), cell lysis buffers and luciferase substrate came from Promega (U.S.A.). Electroporation cuvettes were obtained from BIO-RAD (U.S.A.). SAINT-2^{pp} was synthesised as previously described.[13] DOPE was purchased from Avanti Polar Lipids Inc. (U.S.A.). ^{14}C-labeled chloramphenicol was obtained from Amersham (U.S.A.), n-butyryl-CoA from Fluka (Switzerland) and mixed xylenes from Sigma-Aldrich (The Netherlands). Scintillation fluid (ultima gold XR) came from Packard (U.S.A.). Thin-layer plates were purchased from Merck (Germany) and radiographic films (Kodak X-omat) from Sigma Chemical Co (U.S.A.). pMC1NeoPolyA was used from Stratagene (U.S.A.). Geneticin (G418) was obtained from Boehringer Mannheim (Germany).

Cell culture

Endothelial cells were isolated from human umbilical veins (HUVEC) according to the method of Jaffe *et al.*,[15] except that 0.1 mg/ml chymotrypsin resuspended in phosphate-buffered saline (PBS; 140 mM NaCl, 9.0 mM Na_2HPO_4 and 1.3 mM NaH_2PO_4) was used instead of collagenase. This modification provides a higher yield of EC with fewer contaminating smooth muscle cells.[16] Primary isolates were cultured in 1% gelatin-precoated 25 cm^2 tissue culture flasks at 37 °C under 5% CO_2/95% air. The culture medium consisted of RPMI 1640 supplemented with 20% heat-inactivated human serum, 2 mM L-glutamine, 5 U/ml heparin, 50 µg/ml EC growth factor supplement extracted from bovine hypothalamus,[17] 100 µg/ml streptomycin, and 100 U/ml penicillin. After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml) and split at a 1:3 ratio. Cells were counted with a Coulter counter and used for experiments up to passage five; no differences in transfection rate were observed between these passages.

Electroporation

HUVEC were transfected by electroporation with a Gene Pulser (BIO-RAD) at 200-400 V and 250-960 µF ($n = 1$). Electroporations were performed with 10^6 cells and 10 µg pCAT in 600 µl culture medium in a 0.4 cm cuvette. After electroporation cells were transferred into 100 mm cell culture dishes with 10 ml of culture medium. Optimal voltage and capacity were used for further experiments. Cell concentrations were lowered step-wise from 5×10^6 to 10^2 cells per electroporation ($n = 1$) and a more sensitive reporter system was used (pCMVLuc). When fewer than 10^5 cells were electroporated, they were transferred into 6-well clusters with 2 ml of culture medium.

SAINT-2^{pp}/DOPE transfection

The day before transfection, 2×10^5 HUVEC were transferred into 6-well clusters. The next day the cells were washed twice with Hepes-buffered saline (HBS; 20 mM Hepes, 150 mM NaCl, pH 7.4). Amounts of 10-50 nmol SAINT-2^{pp}/DOPE were mixed with 1 µg pCMVLuc (ratios 10:1 to 50:1) in 1 ml of HBS and added to the cells ($n = 1$). After incubation for 4 h at 37 °C, 1 ml culture medium was added for further incubation overnight. The optimal ratio was used in combination with cell concentrations lowered step-wise from 2×10^5 to 10^2 cells per transfection ($n = 1$).

CAT assay

The following procedure was used to measure chloramphenicol acetyl transferase (CAT) activity.[18] Two days after transfection, cell extracts were made with reporter lysis buffer and cell remnants were removed by centrifugation. The supernatant was incubated for 4.5 h with 75 nCi ^{14}C-labeled chloramphenicol and 25 µg n-butyryl-CoA in Tris-buffer (0.25 M, pH 8.0) at 37 °C.

For liquid scintillation counting (LSC), mixed xylenes were added. The organic phase was transferred to a scintillation vial filled with scintillation fluid and measured in a liquid scintillation counter (Packard). The amount of radioactivity expressed in disintegrations per minute (dpm) correlates with the CAT activity.

For thin layer chromatography (TLC), ethyl acetate was added. The organic phase was dried and in a small volume of ethyl acetate the samples were applied

to the thin-layer plate. After chromatography with chloroform / methanol (19:1), CAT activity was visualised by the intensity of black spots on a radiographic film.

Luciferase assay

To measure luciferase activity,[19] cell extracts were made with cell culture lysis reagent one day after transfection and cell remnants were removed by centrifugation. Luciferase assay substrate was added to the supernatant and luminescence was measured (Lucy-1, Anthos). The amount of luminescence expressed in relative light units (rlu) correlates with the luciferase activity.

Transfection rate

Electroporation and SAINT-2^{pp} / DOPE transfection of 10^5 HUVEC were performed with various concentrations of pMC1NeoPolyA ($n \geq 4$). One day after transfection, cells were provided with fresh culture medium; on the second day they were transferred to a larger surface. G418 was added at a concentration of 400 µg / ml for at least 8 days. Cell doubling of each transfected cell which survived the process caused the formation of one colony. These colonies were counted by light microscopy and the transfection rate was expressed in colony-forming units (1 CFU = formation of one colony when 1 µg plasmid and 10^5 EC were used).

Fluorescent in situ *hybridisation*

EC cultured after transfection with pMC1NeoPolyA were detached from the surface of the culture flask by trypsin / EDTA, subjected to hypotonic treatment with 0.025 M KCl, fixed in methanol-acetic-acid, 3:1 (v / v) and dropped onto slides according to standard procedures for the preparation of metaphase spreads. Fluorescent *in situ* hybridisation (FISH) was performed on the preparations according to standard laboratory protocols using as a probe the biotinylated plasmid pMC1NeoPolyA. Integration of the plasmid was assessed by fluorescence microscopy.

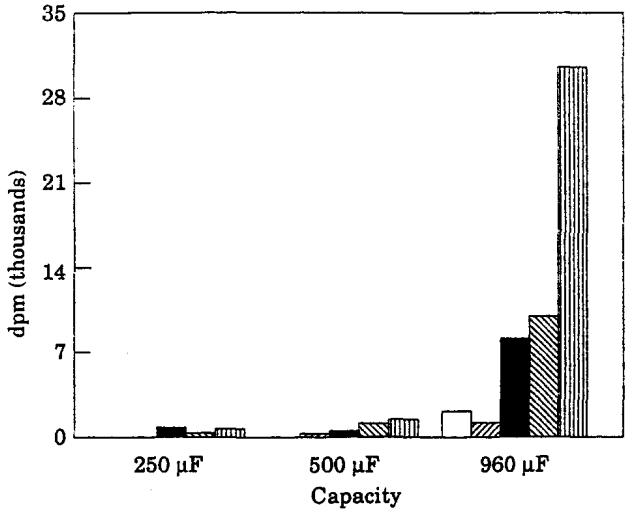

Fig. 1. Transfection by electroporation with pCAT at different voltages and capacities. Electroporation of 10^6 HUVEC with 10 µg pCAT in 600 µl culture medium in a 0.4 cm cuvette at 200, 250, 300, 350 and 400 V; and 250, 500 and 960 µF (n = 1). CAT activity is expressed in dpm. (❑) 200 V; (▨) 250 V; (■) 300 V; (▧) 350 V; (▥) 400 V.

Statistics

The results shown in Fig. 6 are presented as mean values and standard deviations of at least four experiments. To compare the CFU-values at different concentrations of plasmid, the unpaired *t*-test was used.

Results

To determine optimal electroporation conditions for HUVEC, five different voltages (200, 250, 300, 350 and 400 V) were used at three different capacities (250, 500 and 960 µF). Figure 1 shows that an increase of voltage and capacity resulted in an increase of CAT activity. HUVEC even survived 400 V and 960 µF, which resulted in the highest CAT activity. These optimal conditions were used to determine the minimum amount of HUVEC still expressing CAT activity after electroporation. Figure 2 shows that decreasing the amount of cells resulted in a decreased CAT activity. At 10^5 HUVEC almost no CAT activity was left.

SAINT-2^{pp} / DOPE transfections of HUVEC resulted in CAT activities fluctuating around background values, as measured with LSC (results not shown). TLC was used to visualise the product formed by the CAT enzyme. However, no black spots could be detected on the radiographic film in case of SAINT-2^{pp} / DOPE transfections. Even for electroporation, when the highest CAT activity measured by LSC was used, only a very weak spot was present after TLC (results not

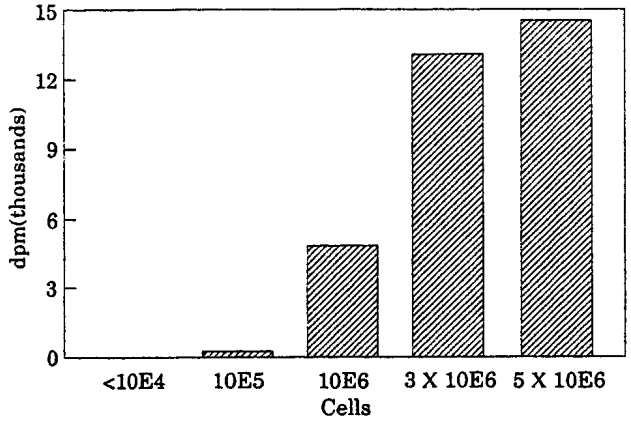

Fig. 2. Transfection by electroporation with pCAT and different amount of cells. Electroporation of 5×10^6, 3×10^6, 10^6, 10^5, 10^4, 10^3 and 10^2 HUVEC with 10 µg pCAT in 600 µl culture medium in a 0.4 cm cuvette at 960 µF and 400 V ($n = 1$). CAT activity is expressed in dpm.

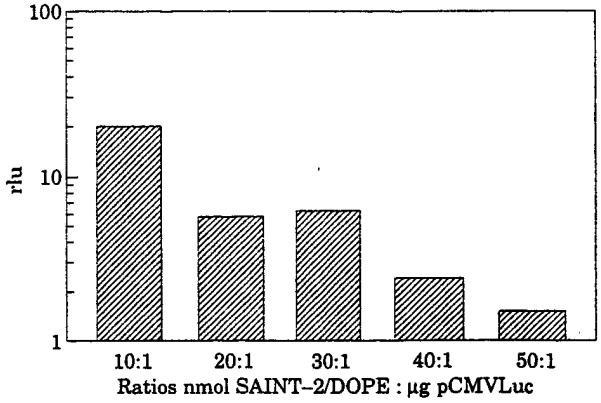

Fig. 4. Comparison of different concentrations SAINT-2^{pp}/DOPE. Transfection of 2×10^5 HUVEC in a 6-well cluster with 10, 20, 30, 40 and 50 nmol SAINT-2^{pp}/DOPE and 1 µg pCMVLuc (ratios 10:1 to 50:1) in 1 ml HBS for 4 h ($n = 1$). Luciferase activity is expressed in rlu.

shown). Therefore a switch was made to a more sensitive reporter system (pCMVLuc).

Electroporation with decreasing amounts of HUVEC was repeated with pCMVLuc instead of pCAT. Figure 3 shows a decrease of luciferase activity when fewer cells were electroporated. Compared with the CAT assay, the luciferase assay was a hundred-fold more sensitive. At 10^3 HUVEC there was still some activity present. In case of SAINT-2^{pp}/DOPE transfections, luciferase activities were much higher than background values. Figure 4 shows that an increasing concentration of SAINT-2^{pp}/DOPE at a constant plasmid concentration resulted in a decreased luciferase activity. At the conditions tested, the highest activity was found when 10 nmol/ml was used at a plasmid concentration of 1 µg/ml (ratio 10:1). This ratio was used to determine the minimum amount of HUVEC still expressing luciferase activity after SAINT-2^{pp}/DOPE transfection.

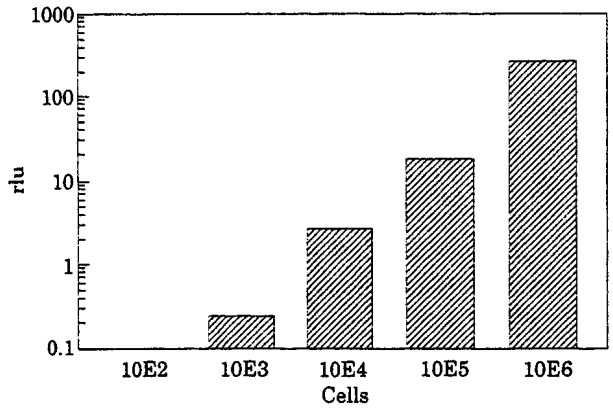

Fig. 3. Transfection by electroporation with pCMVLuc and different amount of cells. Electroporation of 10^6, 10^5, 10^4, 10^3 and 10^2 HUVEC with 10 µg pCMVLuc in 600 µl culture medium in a 0.4 cm cuvette at 960 µF and 400 V ($n = 1$). Luciferase activity is expressed in rlu.

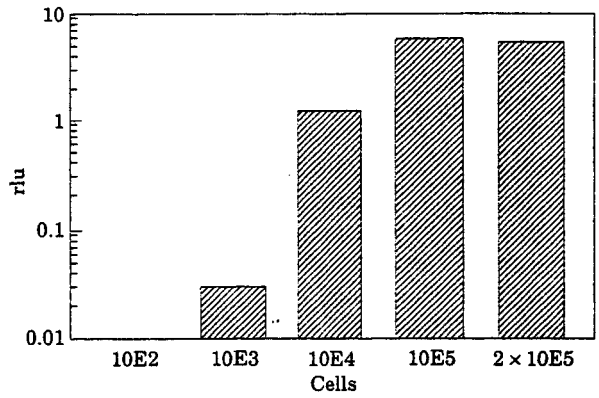

Fig. 5. SAINT-2^{pp}/DOPE transfection with pCMVLuc and different amount of cells. Transfection of 2×10^5, 10^5, 10^4, 10^3 and 10^2 HUVEC in a 6-well cluster with 10 nmol SAINT-2^{pp}/DOPE and 1 µg pCMVLuc in 1 ml HBS for 4 h ($n = 1$). Luciferase activity is expressed in rlu.

Figure 5 shows that a decreasing amount of cells resulted in a decreased luciferase activity. Again, even with only 10^3 HUVEC activity was present. Figures 1-5 show the results of one representative experiment. More experiments were performed, but in transient transfection assays the absolute values on the y-axis cannot be compared between different experiments.

To investigate whether either transfection method has an advantage over the other in forming colonies, pMC1NeoPolyA was used instead of pCMVLuc. More colonies were counted with a higher plasmid concentration for electroporation was used. Although, at

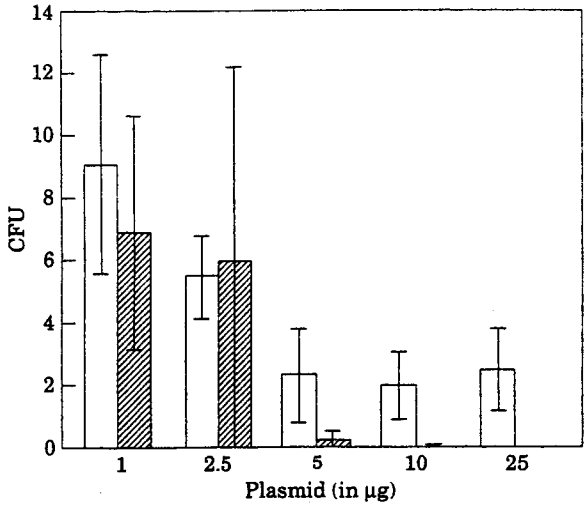

Fig. 6. Comparison of two transfection methods at different plasmid concentrations. Electroporation of 10^5 HUVEC with 1, 2.5, 5, 10 and 25 µg pMC1NeoPolyA. SAINT-2^{pp}/DOPE transfection of 10^5 HUVEC with 1, 2.5, 5 and 10 µg pMC1NeoPolyA. Optimal transfection coditions were used and colonies were counted after selection with G418. Transfection rate is expressed in colony-forming units (CFU = amount of colonies per µg plasmid per 10^5 EC) ± S.D. ($n \geq 4$). (❑) Electr; (▨) SAINT-2.

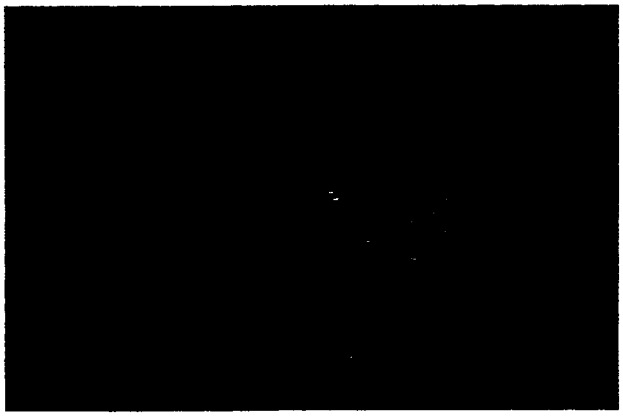

Fig. 7. FISH of the plasmid used for transfection of EC on metaphase chromosomes of transfected EC. Specific hybridisation on a chromosome was detected with fluorescein-avidin (yellow green) in combination with goat anti-avidin. Total DNA was stained with propidium iodide (red).

low plasmid concentrations (1 and 2.5 µg) Figure 6 shows a higher CFU (significant, $p<0.05$). In case of SAINT-2^{pp}/DOPE transfection colonies were counted only at low plasmid concentrations. At 5 and 10 µg plasmid, the transfection mixture, which also contained 50 and 100 nmol synthetic amphiphiles (ratio 10:1), became toxic for the cells.[13,14] Figure 6 shows that the CFU for both methods was about the same at low concentrations of plasmid (not significant, $p<0.1$). Figure 7 shows, by using FISH, that integration of the plasmid occurred in one chromosome per transfected EC.

Discussion

Both electroporation and lipofection with SAINT-2^{pp}/DOPE appeared to be efficient transfection methods for EC. Electroporation should be performed at 400 V and 960 µF and similar optimal conditions have been reported before by Kotnis *et al.*[11] In the case of SAINT-2^{pp}/DOPE transfection the ratio 10:1 (nmol SAINT-2^{pp}/DOPE:µg plasmid) should be used instead of higher ratios, in order to get better transfection. Synthetic amphiphile mediated transfections are usually performed with 1 µg of plasmid[13,14] and electroporations with up to 50 µg. This study therefore started with 1 µg for SAINT-2^{pp}/DOPE transfection and 10 µg for electroporation. When we used a sensitive transient expression assay (luciferase instead of CAT), we were able to detect activity with both transfection methods in as few as one thousand EC. This is important for the production of functional endothelial cell lines from various human blood vessels, because only small amounts of material are available. If isolation results in only a few EC, transfection can be performed shortly after isolation, when it can be assumed that most of the specific EC functions are still present.

The plasmids used contained the reporter genes chloramphenicol acetyltransferase or luciferase, which cannot be used to determine the percentage of transfected cells. As production of cell lines requires the outgrowth of colonies, both transfection methods were also compared using the neomycin resistance gene. When only a few cells are available and there is no limitation for the use of high plasmid concentrations, electroporation provides for the highest yield of colonies. At low plasmid concentrations the transfection rate expressed in CFU was within the same range. FISH of the plasmid used for transfection of EC on metaphase chromosomes of transfected EC was performed after long-term culture and showed that integration of the plasmid occurred after transfection in both ways. So SAINT-2^{pp}/DOPE mediated lipofection appeared to be as effective as electroporation.

In conclusion, this study shows that as few as 1000 EC can be transfected by electroporation and with SAINT-2^{pp}/DOPE. Both methods result in stable integration of the plasmid and at low plasmid concentrations, they also have the same transfection rate. Transfection of small numbers of EC shortly after isolation will reduce the risk of losing original functions before transfection. This is important to obtain functional endothelial cell lines from various human blood vessels for research purposes such as thrombosis in vascular diseases. Transfections of adult venous and arterial cells are in progress.

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