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Optimisation of Gene Transfer into Vascular Endothelial Cells using Electroporation

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Objectives: *We have examined the conditions required to obtain optimum transfection efficiencies for human umbilical vein endothelial cells by transduction with a plasmid conferring neomycin resistance.*
Materials and Methods: *Preliminary studies examined the effects of electric discharges using the Biorad Gene Pulser on endothelial cells. Post-electroporation, there was a significant decrease in cell survival with increasing voltages (100–400 volts; p = 0.03), capacitances [125–960 microFarads (μF); p = 0.02], number of electric pulses (1–2; p = 0.03) and decreasing cell concentrations (p = 0.01). The optimal cell concentration was 3×10^6 cells/ml. Transfection studies utilised the neomycin resistance expressing plasmid, pTCF; transfectants were selected with the neomycin analogue G-148.*
Results: *Electro-transfection was optimised with increasing voltages (p = 0.02) and capacitances (p = 0.01) using a single pulse. Optimal transfection was obtained using 400 volts with a capacitance of 960μF using a single pulse; the median transfection efficiency was 10%. Transduced endothelial cells stably expressed the plasmid for 12 days and at least two cell passages.*
Conclusions: *The results indicate that endothelial cells can be efficiently transduced by electroporation to stably express an introduced gene. This may have important implications in vascular surgery.*

Key Words: Electroporation; Endothelial cells; Transfection.

Introduction

Much attention has been directed towards the vascular endothelium as a potential vehicle for gene transfer, aimed at improving the patency of vascular prostheses[1–4] and treating gene defects.[2,5–6] The strategic location of endothelial cells makes them an attractive target for the delivery of gene products with paracrine effects.[6] The luminal release of exogenous proteins by genetically modified endothelium has been proposed as a means of treating thrombosis, platelet aggregation, vasoconstriction and smooth muscle cell proliferation[7] following arterial reconstruction. The introduction of foreign genes into endothelial cells using lipofection[2,7,8] and retroviral transfection[9–12] has been extensively studied, with little attention as yet addressed towards electroporation as a mechanism of transfection.

Electroporation is a rapid, simple and efficient method for introducing foreign DNA into mammalian cells[13–17] and has been reported to produce higher transfection efficiencies than calcium phosphate and DEAE-dextran mediated transduction procedures.[18] Electroporation has often been termed electric field mediated gene transfer[17] since it uses an electric field to make the cell membrane reversibly permeable.[14,19] The cell membrane acts as an electrical capacitor which is unable (except through ion channels) to allow current to flow. Following exposure to an electric discharge, polarisation of the membrane leads to the formation of a potential difference[19] which if large enough to exceed a threshold value leads to pore development in the cell membrane. The pores may be of a sufficient size to allow the transmembrane passage of macromolecules such as DNA.[20] The reclosing of the permeable cell membrane occurs naturally; but if the combination of the magnitude and the duration of the electric field exceed a threshold value, the cell membrane is irreparably damaged. In this study, electroporation was performed using a gene pulser and a capacitance extender (Biorad,

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Hemel Hempstead, U.K.) capable of delivering a high voltage of a long duration. The maximum voltage and capacitance achievable by this electroporator were 400V and 960μF respectively. The plasmid pTCF[21] which confers neomycin resistance was used for the transfection experiments.

The most important parameters for successful electroporation are the electric field and the time constant (duration of the electric discharge). This study aimed to optimise electro-transfection of endothelial cells by determining an electric field and time constant that would lead to most efficient gene transfer. The electric field is determined by the voltage and the distance between the electrodes of the electroporetic cuvette; since the latter was always constant, the voltage was varied in order to find an optimum electric field. Similarly the time constant is determined by the capacitance and the resistance of the apparatus and as the resistance was always constant, the capacitance was varied in order to define an optimum time constant.

It has been proposed that electroporation may irreparably damage cells and so the ability of human endothelial cells to survive electric pulses was initially studied. Based on these initial viability experiments, the range of values for the voltage and capacitance that could have resulted in electro-transfection were then used for the transfection experiments. Preliminary studies involved defining an optimum voltage for electro-transfection of endothelial cells with a constant capacitance. The optimum voltage was then constantly maintained and the capacitance varied in order to further optimise transfection. The use of multiple electric pulses to optimise electro-transfection has been advocated and was also studied.[22, 23] Finally the stability of expression of the plasmid DNA was assessed.

Materials and Methods

Endothelial cell harvest and culture

Endothelial cells were procured under sterile conditions from human umbilical cords within 24 hours of delivery using a method modified from Jaffe *et al.*[24] Identification of endothelial cells was confirmed by their characteristic cobblestone appearance and by staining with rabbit anti-human von Willebrand Factor antibody (Dakopatts, Glostrup; Denmark) and a mouse monoclonal antibody specific for thrombomodulin (QB-END/40.1 — Quantum Biosystems Ltd, U.K.). Cells were cultured in T25, 80 and 175 tissue culture flasks (Nunclon, Kamstrup; Denmark) with Medium 199 (ICN, High Wycombe, U.K.) containing 20% fetal calf serum (FCS — Sera Lab, Lot 001010, U.K.), 100U/ml streptomycin, 100U/ml penicillin (NBL, Northumbria, U.K.) at 37°C, 95% air and 5% CO_2. Endothelial cells were passaged at confluence using 0.1% trypsin in 0.02% EDTA (ICN, High Wycombe, U.K.) in a ratio of 3:1. All experiments were performed using endothelial cells at third passage.

Electroporetic procedure

Endothelial cells harvested from culture flasks with trypsin/EDTA were washed with MEM + 5% FCS by centrifugation at 300*g*, 4°C for 7 minutes. Viability was assessed by trypan blue exclusion (Sigma, Poole, U.K.) using a haemocytometer (Weber, Lancing, U.K.). Viability was greater than 95% in all cases. Endothelial cells at a known concentration were then centrifuged (under the same conditions as above) twice; firstly with 5 ml of MEM and then with 10 ml of phosphate buffered saline (PBS). The final endothelial cell pellet was resuspended in 0.8 ml of PBS (the electroporetic volume), and transferred to a plastic cuvette (Biorad, Hemel Hempstead, U.K.). The cuvette contained two aluminium electrodes between which the electric discharge passed. Endothelial cells were placed between the electrodes and were therefore subjected to the electric current as required. The capacitance and voltage to be used were selected and the plasmid DNA added at this stage. Electroporation was performed using a Biorad electroporator (gene pulser and capacitance extender) under a sterile hood (Gelaire BSB, U.K.) at 24°C. Following the electroporetic procedure, the cells were immediately transferred into fresh culture medium and incubated at 37°C, 95% air and 5% CO_2.

Plasmid DNA

The plasmid pTCF which contains a gene for the enzyme conferring neomycin resistance, aminoglycosidephosphotransferase (AGPT),[21] was purified using caesium choride gradient centrifugation.[25] The enzyme AGPT is responsible for breaking down G-418 which inhibits protein synthesis by interfering with the codon-anticodon relationship.[26] Endothelial cells that incorporate pTCF will be able to translate the AGPT gene and therefore prevent G-418 from exerting its inhibitory actions on protein synthesis. Non-

transfected endothelial cells will be subjected to the toxicity of G-418 and will therefore die when this neomycin analogue is added to their culture medium.

Viability experiments

In order to investigate the effects of varying the strength and duration of electric charge on human endothelial cell survival, endothelial cells at passage three were subjected to electroporation using different voltages and capacitances using the same electroporetic procedure described above. No plasmid DNA was utilised during these viability experiments. Following the electroporetic procedure, endothelial cells were placed in a tissue culture flask with complete culture medium and incubated at 37°C in a 95% air and 5% CO_2 atmosphere. After incubation for 24 hours, the electroporated endothelial cells were assessed for cell survival using trypan blue exclusion. All experiments were repeated three times.

(a) The effect of cell concentration on cell survival (%) post-electroporation. Endothelial cells at the third subculture were harvested from culture flasks using 0.1% trypsin in 0.02% EDTA and electroporated at concentrations of 1, 3 and 5 $\times$ 10^6 cells/ml using a single pulse of 300 volts with a time constant of 12.6 ms (capacitance 960μFarads).

(b) The effect of voltage on cell survival (%) post-electroporation. The response of endothelial cells to voltages of 100, 200, 300 and 400 volts potential difference were assessed. Using the optimum cell concentration of 3 $\times$ 10^6 cells/ml, defined from the previous experiment, endothelial cells were electroporated with the above voltages and a constant capacitance of 960μF yielding a time constant of 12.6 ms.

(c) The effect of time constant on cell survival (%) post-electroporation. Differing time constants for the electric discharge were achieved by varying the capacitance. Endothelial cells at a concentration of 3 $\times$ 10^6 cells/ml were electroporated at 300 volts using capacitances of 125, 250, 500 and 960μF leading to time constants of 2.5, 4.3, 6.8 and 12.6 ms respectively.

(d) The effect of multiple electric pulses on cell survival (%) post-electroporation. Endothelial cells at a concentration of 3 $\times$ 10^6 cells/ml were electroporated using two electric pulses of 300 volts and 960μF capacitance (time constant of 12.6 ms) to determine the ability of human endothelial cells to survive a second pulse. The gene pulser (Biorad, Hemel Hempstead, U.K.) was unable to deliver consecutive pulses automatically and subsequently this was performed manually. To minimise any inaccuracies due to the time gap between pulses the second pulse was delivered immediately after the first pulse but a delay of 5 seconds was still incurred due to recharging of the capacitor.

Transfection studies and G-418 selection

The viability results were used to set the parameters for optimising transfection. Electro-transfection was performed at a concentration of 3 $\times$ 10^6 cells/ml with voltages between 200–400 volts and capacitances from 125–960μF. Cells were transfected with the test plasmid pTCF. The selection of transfectants was performed using the neomycin analogue G-418 (GIBCO, Paisley, U.K.) at a concentration of 50μg/ml which kills all non-transfected endothelial cells by day 10 (as previously established in our laboratory). Endothelial cells were electroporated at day 0 with 24μg/ml of the plasmid pTCF (a concentration of plasmid DNA commonly used in electroporation experiments)[15] and the culture medium replaced on day 2; on consecutive days thereafter, the existing culture medium was replaced with fresh culture medium supplemented with 50μg/ml G-418. Three sets of controls were used: endothelial cells alone; endothelial cells electroporated with no pTCF and finally cells not electroporated but containing pTCF in their culture medium. Transfection experiments were assessed on day 9 for the number of viable transfected cells as determined by trypan blue exclusion. The number of transfected endothelial cells was divided by the initial cell number to attain a transfection efficiency (%).

(a) The effect of voltage on transfection efficiency of endothelial cells. Endothelial cells at a concentration of 3 $\times$ 10^6 cells/ml were exposed to voltages of 200, 300 and 400 volts with a constant time constant of 12.6 ms (960μF capacitance). The transfection efficiency (TE) was calculated on day 9. The voltage transfection experiment was repeated four times.

(b) The effect of time constant on transfection efficiency of endothelial cells. Capacitances of 125, 250, 500 and 960μF with time constants of 2.5, 4.3, 6.8 and 12.6 ms respectively and a constant voltage of 400 volts were

used to assess the effect of changes in the time constant on transduction efficiency. Endothelial cells at 3×10^6 cells/ml were electro-transfected using the above parameters with the controls as previously described. All cells underwent the same selection procedure and the TE was calculated on day 9. A voltage of 400 volts was chosen because it produced the optimum TE as determined from the previous experiment. The experiment was again repeated four times.

(c) The effect of multiple electric pulses on transfection efficiency of endothelial cells. In order to attempt to optimise transfection even further, the optimum parameters of 400 volts and 960μF capacitance as determined above were used for electroporation but this time two electric discharges were passed through the endothelial cells. Electro-transfection was also attempted with two electric pulses at 200 and 300 volts at 960μF. All experiments were repeated four times.

Stability of expression of the plasmid pTCF

In order to determine whether the introduced plasmid pTCF was being stably expressed, endothelial cells electro-transfected with pTCF and then selected with 50μg/ml G-418 for 9 days were plated at a concentration of 5×10^4 cells/ml per well in a 24 well plate (Nunclon, Kamstrup, Denmark). The transfected endothelial cells were cultured with or without 50μg/ml G-418. Transfected cells grown in culture medium without G-418 acted as a control together with non-electroporated endothelial cells grown with or without 50μg/ml G-418. Each day, the endothelial cells in 4 wells were harvested and the viable cell concentration determined by trypan blue exclusion. If the plasmid pTCF was stably expressed, transfected cells grown with 50μg/ml G-418 would survive due to expression of AGPT, whereas non-transfected endothelial cells would fail to survive.

The number of cell passages that the introduced plasmid pTCF was expressed was also studied. Transduced endothelial cells were grown on culture medium in T25 tissue culture flasks with or without 50μg/ml G-418 and passaged to confluence. Control non-electroporated endothelial cells were also either exposed to G-418 or grown with culture medium alone. The longer the expression of the plasmid, the more passages but the transfected cells would survive when grown with culture medium and 50μg/ml G-418. When transfected cells grown with G-418 died but transfected cells grown without G-418 survived, loss of or inactivation of the gene for AGPT was assumed to have occurred.

Statistics

Statistical analysis of results was performed using the Minitab Release 8.1 statistical programme (Minitab Inc, Pennysylvania, U.S.A.) on a Macintosh LC personal computer (Apple Computer Inc, California, U.S.A.). The results are presented as median values with 95% confidence intervals where appropriate. The statistical tests used were the Mann-Whitney U Test and linear regression analysis.

For the data regarding the stability of expression of the plasmid DNA, significance was assessed by calculating the areas under the curves, which represented the total number of cells over the period of study and performing Mann-Whitney U Test analyses on the data.

Results

Viability studies

(a) The effect of cell concentration on cell survival post-electroporation. This experiment demonstrated that there was a significant increase in cell survival (%) with increasing cell concentrations (Fig. 1) (r^2 = 82.8%, p = 0.001; linear regression analysis). From these results (Fig. 1), it was concluded that transfecting with 3×10^6 cells/ml would yield optimum transfection efficiencies as there was no significant difference in the cell survival (%) when electroporating with concentrations of 3×10^6 and 5×10^6 cells/

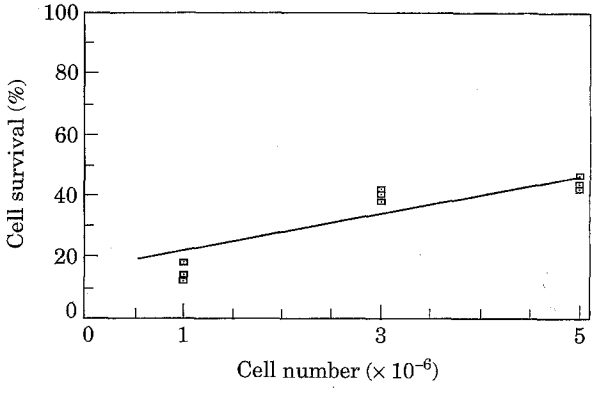

Fig. 1. Graph showing the effect of varying cell concentrations on cell survival (%) after electroporation. Individual points are plotted with the best fit linear regression line.

ml (W = 6.5, p = 0.13; Mann-Whitney U Test). In transfection studies a cell concentration of 3×10^6 cells/ml was utilised as this represented a balance between maximum cell survival and the quantity of cells required for individual experiments.

(b) The effect of voltage on cell survival post-electroporation. As the transfection voltage increased from 100–400 volts, there was a significant decrease in endothelial cell survival (%) (Fig. 2; r^2 = 92.4%, p = 0.03. Linear regression analysis).

(c) The effect of time constant on cell survival post-electroporation. Increasing capacitances and thus time constants led to a significant decrease in cell survival post-electroporation (Fig. 3; r^2 = 82.8%, p = 0.02. Linear regression analysis). The experiment showed that endothelial cells could survive voltages with capacitances up to 960μF.

(d) The effect of multiple electric pulses on cell survival post-electroporation. Pulsing endothelial cells twice as compared to once resulted in a decrease in cell survival (Fig. 4; W = 26, p = 0.03. Mann-Whitney U Test). This result demonstrated that endothelial cells were more sensitive to the introduction of a second pulse as compared with only one pulse. Nevertheless, endothelial cells were able to survive a second pulse and so this was used in the transfection experiments.

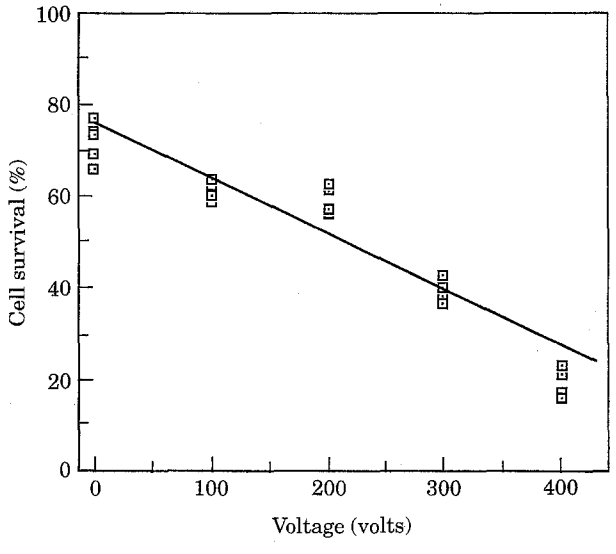

Fig. 2. Graph showing the effect of voltage on endothelial cell survival (%) post-electroporation. The data is presented as a linear regression line showing the best fit.

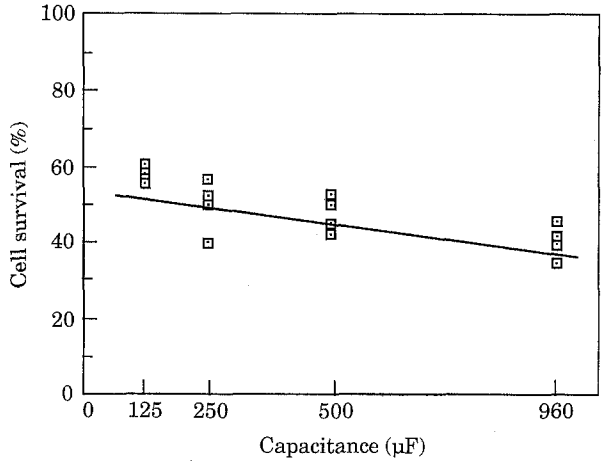

Fig. 3. Graph demonstrating the effect of varying capacitances and thus time constants on endothelial cell survival following electroporation. Individual points are plotted with the best fit linear regression line.

Transfection studies

(a) Morphology of transfected endothelial cells. Transfected endothelial cells displayed the characteristic cobblestone pattern of endothelial cells under phase contrast microscopy. The transfected cells formed close colonies of cells.

(b) The effect of voltage on transfection efficiency. As the voltage was increased, electro-transfection became optimised (Fig. 5; r^2 = 60.2%, p = 0.002. Linear regression analysis). Transfection was optimal at 400 volts with a median TE of 10%. Comparable TE's were obtained using 300 volts but at 200 volts the TE was reduced (Fig. 5). All experiments were performed at

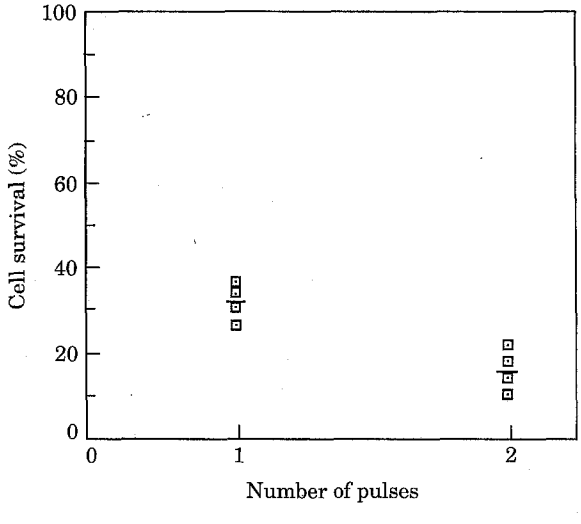

Fig. 4. Graph showing the effect of one or two electric pulses on endothelial cells. The median cell survival at one and two pulses is shown.

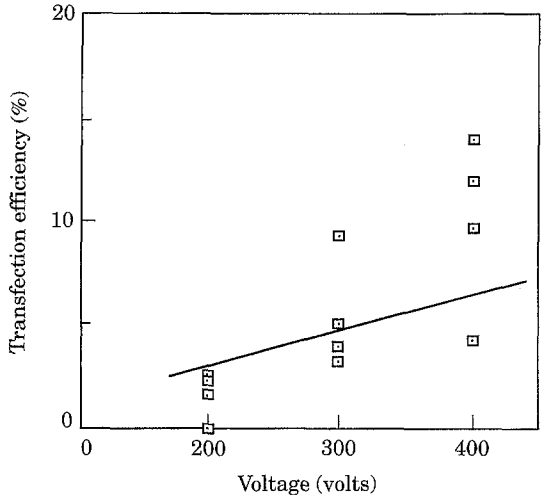

Fig. 5. Graph displaying the effect of changes in voltage on the transfection efficiences of endothelial cells. Individual points are plotted with the best fit linear regression line. A voltage of 400 volts produced the optimum transfection efficiency with a median value of 10%.

960μF capacitance to allow voltage to be the only variable.

(c) The effect of capacitance on transfection efficiency. Decreasing capacitances (time constants) produced a significant reduction in TE (Fig. 6; r^2 = 53.7%, p = 0.001. Linear regression analysis). Optimal transfection efficiencies were obtained using capacitances of 960μF (Fig. 6).

(d) The effect of multiple electric pulses on transfection efficiency. Following optimising transfection with a single pulse at 400 volts and 960μF, transfection was attempted with the same parameters but with two pulses. At 400 volts double pulse, the endothelial cells were unable to survive the electroporation; 24 hours after the procedure all endothelial cells had died. With double pulsing at 300 volts there was initial cell survival but after 72 hours the endothelial cells died. Finally the double pulse was attempted at 200 volts, the endothelial cells survived the G-418 supplement on days 4 and 6 but by day 7, the cells had died and no transfectants remained.

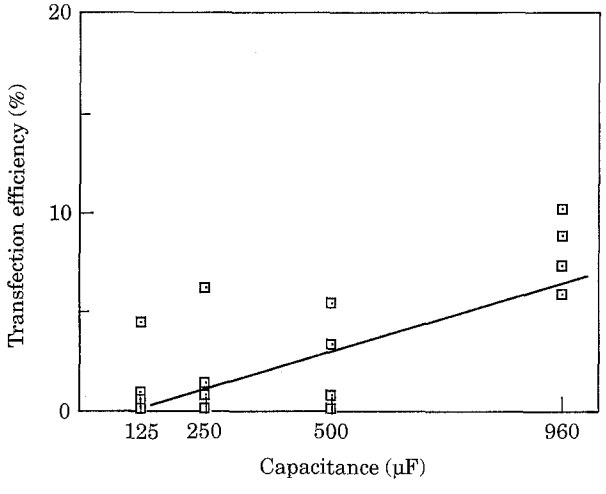

Fig. 6. Graph showing the transfection efficiencies of endothelial cells with varying capacitances up to 960μFarads. Optimum transfection was gained using 960μFarads. The data is plotted as a linear regression showing the line of best fit.

(e) The stability of expression of the plasmid. Endothelial cells transfected with the plasmid pTCF and grown with 50μg/ml G-418 demonstrated no significant difference in reproductive capacity compared with transfected cells cultured without G-418 up to day 12 (Fig. 7; W = 103.5, p = 0.94. Mann-Whitney U Test). After 12 days, there was a decrease in cell survival for the G-418 grown transduced cells whereas the transduced cells grown without G-418 continued to retain their viability.

Non-transfected control endothelial cells grown with 50μg/ml G-418 failed to survive after day 10 but displayed similar proliferative rates to the transfected cells up to day 5 (Fig. 7). Control endothelial cells grown without G-418 maintained viability past 20 days. There was no significant difference in the growth rates of transduced cells grown with or

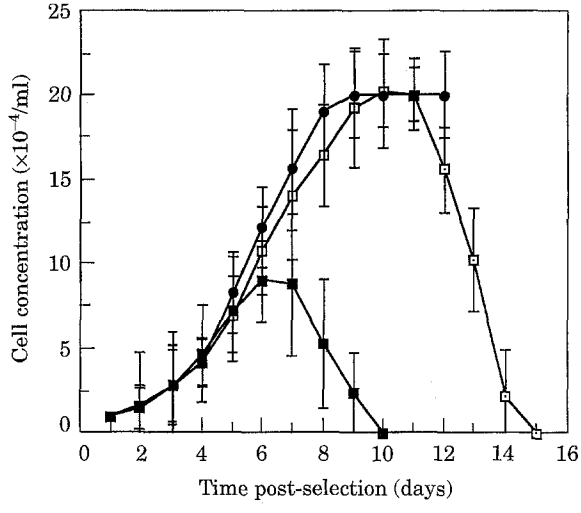

Fig. 7. Graph showing the stability of expression of the plasmid pTCF after electro-transfection of endothelial cells. The transduced endothelial cells were grown on either culture medium alone (control) or with culture medium supplemented with 50μg/ml G-418. A further control of non-electroporated endothelial cells grown with culture medium and 50μg/ml G-418 was also used. The points are plotted as median values with standard error bars. (□) transfected cells 50μg/ml; (•) transfected cells; (■) control cells 50μg/ml.

without G-418 up to day 12 ($W = 103.5$, $p = 0.94$; Mann-Whitney U Test). This result shows that the introduced plasmid pTCF was stably expressed for up to 12 days.

The number of cell passages that the plasmid pTCF was expressed was assessed by growing transfected cells on culture medium containing 50μg/ml G-418. At confluence, the transfected cells were passaged and allowed to proliferate to confluence again. Transfected endothelial cells grown with culture medium supplemented with 50μg/ml G-418 were able to survive the first passage and rapidly repopulated the tissue culture flask at passage 1. At passage 2, the transfected endothelial cells again survived and grew to confluence but by passage 3, the transfected cells grown with G-418 gradually slowed in growth and eventually died. Since transfected cells grown without G-418 survived past passage 3, it was concluded that the introduced plasmid had been lost by this passage. Control untransfected endothelial cells grown with 50·g/ml G-418 were unable to survive the first passage and became non-viable without attaining confluence.

Discussion

Electroporation has been used to deliver foreign genes to endothelial cells.[22–23] This study aimed to optimise electro-transfection by investigating the factors most essential for gene transfer and manipulating those factors to achieve optimisation of transduction. Viability experiments were initially performed to identify the critical parameters and the transfection studies utilised the viability results to optimise transduction.

The most crucial parameters for electro-transfection are the cell concentration, electric field, time constant and the number of electric pulses. The effects of changes in these parameters on endothelial cell survival post-electroporation were investigated. Prior to transfecting endothelial cells with the plasmid pTCF, it was necessary to determine an optimum cell concentration that would result in an acceptable cell survival (%) post-electroporation. Since cell types differ in their ability to respond to an electric pulse,[19] it follows that a particularly susceptible cell type may benefit from increased cell numbers prior to electroporation. The results (Fig. 1) showed a significant increase in cell survival with increasing cell concentrations up to 3×10^6 cells/ml. There was no significant difference in the cell survival when electroporating with cell concentrations of 3 and 5×10^6 cells/ml. Chu *et al.*[15] attempted electro-transfection into human fetal lung fibroblasts with cell concentrations ranging from 10^6 to 10^7 cells/ml and reported similar TE regardless of the initial cell density. The results from this viability experiment showed that transduction would be optimal when electroporating with 3×10^6 cells/ml and that electroporating with 1×10^6 cells/ml would yield too few transfectants.

As the voltage increased the cell survival significantly decreased (Fig. 2). This has been reported[19] and may be explained by the principle that when high voltages are passed through cells, the potential difference created exceeds the critical threshold for irreparable damage of the cells. At lower voltages, much fewer cells are irreparably damaged and consequently, cell survival is significantly higher. The voltage viability experiments demonstrated that endothelial cells were capable of maintaining viability when exposed to voltages as high as 400 volts (maximum obtainable on gene pulser) and for that reason the transfection studies used voltages up to 400 volts.

As the time constant increased, cell survival significantly decreased (Fig. 3). This shows that capacitance is an important factor for electroporation. Longer time constants lead to wider pores and longer duration of the pores and although this increases the possibility of DNA entering, it simultaneously decreases the cell survival as shown by the viability results.

The increased cell loss using two electric pulses as compared with one (Fig. 4) stems from the principle that the longer the pulse, the stronger the electric field and the more energy that is dissipated across the sample. Increased energy leads to an increase in cell death since many more cells are irreparably damaged. Although the double pulse viability experiment showed it was a feasible option, it was found that long term viability was severely impaired.

More extensive membrane dissociation and pore development ensued with increased voltage but antagonistically, greater cell loss occurred (Fig. 2). The increased pore formation will result in increased transfection but the reduction in cell viability leads to a decrease in the number of transfectants. Optimising transfection necessitates finding a voltage that produces the largest number of viable and reversibly permeable cells. Electro-transfection was optimal at 400 volts. Since this value produced the greatest cell loss, it would be assumed that it would not produce optimal TE. The fact that 400 volts was optimal showed that its effect on DNA entry must have been potent enough to combat the large reduction in cell viability. Electroporating with 200 and 300 volts which both produced higher cell survivals yielded lower transfectants and therefore it can be assumed that their effect on DNA entry was much less than 400 volts.

As the capacitance and time constant increased, TE was optimised. Wider pores and longer durations of the pores occur with increasing time constants and therefore DNA, present in the buffer medium has a much larger chance of entering the intracellular compartment. At lower capacitances the pores are smaller and open for a shorter period and therefore TE was not optimised even though the cell survival was higher (Fig. 3) as compared with the larger capacitances. Transfecting endothelial cells using two pulses at 960μF and 200, 300 and 400 volts was unsuccessful. The overall conclusion from the double pulse experiments must suggest that the use of multiple electric pulses is less efficient than single pulse electrotransfection. This has also been reported with mammalian cells.[15] Although multiple pulse electroporesis increases the number of pores, it simultaneously increases the energy dissipation across the cells and leads to a situation where the number of irreparably damaged cells far outweighs the number of viable and reversibly permeable cells. Multiple pulse electrotransfection of endothelial cells has been successful,[22–23] different electroporators were used in these studies and this most probably accounts for the difference in results. The machine used in this study was not able to deliver the second pulse automatically and therefore the second pulse had to delivered by hand; this may account for the failure to electroporate when using two pulses.

If electro-transfected endothelial cells are to be used for seeding of vascular grafts, it is essential that they stably express the introduced gene. Transient expression lasting only 48 hours would not permit clinically effective quantities of the introduced gene to be expressed. Endothelial cells transfected with the plasmid pTCF stably expressed the AGPT gene for neomycin resistance for 12 days and at least two passages. It was concluded that electroporation is an efficient method for introducing foreign DNA into endothelial cells and the optimal parameters for DNA entry have been identified. Endothelial cells that have been electro-transfected with the gene for tissue plasminogen activator (t-PA) may be used to treat thrombosis occurring after balloon angioplasty and endarterectomy. A gene such as t-PA is only required initially to prevent early thrombosis, overexpression of t-PA may lead to degradation of the basement membrane and the initiation of smooth muscle cell proliferation leading to intimal hyperplasia,[27] a cause of intermediate and late graft failure. The intermediate length of expression of the introduced plasmid obtained by the electroporetic conditions outlined in this study may therefore be optimal for gene transfer.

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References

1 Callow AD. The vascular endothelial cell as a vehicle for gene therapy. *J Vasc Surg* 1990; **11**: 793–798.
2 Powell JT, van Zonneveld AJ, van Mourik JA. Gene transfer into specific vascular cells. *Eur J Vasc Surg* 1992; **6**: 130–134.
3 Dichek DA, Neville RF, Zwiebel JA *et al.* Seeding of intravascular stents with genetically engineered endothelial cells. *Circulation* 1989; **80**: 1347–1353.
4 Wilson JM, Birinyi LK, Salomon RN, Libby P *et al.* Implantation of vascular grafts lined with genetically modified endothelial cells. *Science* 1989; **244**: 1344–1346.
5 Nabel EG, Nabel GJ. Gene transfer and cardiovascular disease. In: Topol EJ ed. *Textbook of International Cardiology,* Philadelphia: WB Saunders. 1989; 176.
6 Nabel EG, Plautz G, Nabel GJ. Gene transfer into vascular cells. *J Am Coll Cardiol* 1991; **17**: 189B–194B.
7 Powell J, Klausse-Bos JM, van Mourik JA. The uptake and expression of factor VIII and reporter genes by vascular cells. *Febs-Letts* 1992b; **303** (2–3): 173–177.
8 Nabel EG, Plautz G, Nabel GJ. Site specific gene expression *in vivo* by direct gene transfer into the arterial wall. *Science* 1990; **249**: 1285–1288.
9 Nabel EG. Direct gene transfer into the arterial wall. *J Vasc Surg* 1992; **15**: 931–932.
10 Dichek DA. Retroviral vector mediated gene transfer into endothelial cells. *Mol Biol Med* 1991b; **8**: 257–266.
11 Kahn ML, Lee SW, Dichek DA. Optimisation of retroviral vector-mediated gene transfer into endothelial cells *in vitro. Circ Res* 1992; **71**: 1508–1517.
12 Zwiebel JA, Freeman SM, Kantoff PW *et al.* High level recombinant gene expression in rabbit endothelial cells transduced by retroviral vectors. *Science* 1989; **244**: 220–222.
13 Potter H. Gene transfer by electroporation. *Bio-Rad Bull* 1988; **1346**.
14 Knutson JC, Yee D. Electroporation: parameters affecting the transfer of DNA into mammalian cells. *Anal Biochem* 1987; **164**: 44–52.
15 Chu G, Hayakawa H, Berg P. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res* 1987; **15**: 1311–1326.
16 Emery DW, Smith S, Sibley CH. A simple protocol for optimising electroporation transfection of mammalian cells; parameters to consider. *J Methods Cell Mol Biol* 1989; **1**: 153–164.
17 Spandidos DA. Electric field-mediated gene transfer (electroporation) into mouse Friend and human K562 erythroleukaemia cells. *Gene Anal Tech* 1987; **4**: 50–56.
18 Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1982; **7**: 841–845.
19 Shigekawa K, Dower WJ. Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. *Biotechniques* 1988; **6**.
20 Knight DE, Scrutton MC. Gaining access to the cytosol: The technique and some applications of electropermeabilisation. *Biochem J* 1986; **234**: 497–506.
21 Grosveld F, Lund T, Murray EJ, Mellor AL, Dahl HH, Flavell RA. Construction of cosmid libraries which can be used to transform eukaryotic cells. *Nucleic Acids Res* 1982; **10**: 6715–6732.
22 Fickling SA, Tooze JA, Whittley GSt.J. Characterisation of human umbilical vein endothelial cell lines produced by transfection with the early region of SV40. *Exp Cell Res* 1992; **201**: 517–521.

23 Bidawid-Woodroffe S, Sullivan-Tailyour G, Roig-Farran P, Garnett HM. Increased cytomegalovirus infection of human fibroblast and endothelial cells by electroporation. *J Virol Methods* 1992; **38**: 167–174.
24 Jaffe E, Neumann R, Becker C, Munck R. Culture of human endothelial cells derived from umbilical veins. Identification by morphological and immunological criteria. *J Clin Invest* 1973; **52**: 2754–2756.
25 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning.* A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989; **1**: 1.21–1.53.
26 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning.* A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989; **3**: 16.3–16.29.
27 Clowes AW, Clowes MM, Au YP, Reidy MA, Belin D. Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ Res* 1990; **67**: 61–67.

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