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Experimental study of recombinant eukaryotic expression vector of human eNOS in ECV304

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Summary

Background and purpose: Gene transfer with recombinant non-viral vectors encoding vasodilator proteins, such as endothelial nitric oxide synthase (eNOS), may be a preferential choice in gene therapy of artery restenosis following angioplasty, stent or anastomosis. However, the transfection rate of a non-viral vector, the harmful effects of eNOS transfection on endothelial cells (EC) and the control release of nitric oxide (NO) have been controversial. We designed the eukaryotic expression vector pcDNA3.1-eNOS to study the regulated expression of eNOS (in the presence of various chemical agents) and to evaluate the exogenous NO effect on EC proliferation in vitro.

Methods: The full-length human eNOS cDNA was inserted into the EcoRI cloning site of the pcDNA3.1 expression plasmid and the eNOS direction was tested by restriction enzyme digestion with XhoI to construct recombinant pcDNA3.1-eNOS. After co-transfection of pcDNA3.1-eNOS with pcDNA3.0-EGFP mediated by cationic liposomes into Human umbilical vein endothelial cells (ECV304), the transfection rate and the effect on ECV304 proliferation were calculated by fluorescence microscopy and flowcytometry. eNOS mRNA and protein were detected by reverse transcription-PCR (RT-PCR) and immunofluorescence, respectively. The eNOS activity, NO release and changes of the relevant cells growth curve were assessed after treating the transfected cells with four independent factors including ie Ca^{2+}, L-arginine (L-Arg), Ethylene Diamine Tetraacetic Acid (EDTA) and N-nitro-L-arginine methylester (L-NAME). In addition, we examined the non-transfected cells status by isolated sodium nitroprusside (SNP) treatment.

Result: eNOS cDNA was inserted into pcDNA3.1 in the proper direction. RT-PCR analysis showed that pcDNA3.1-eNOS transfected cells could express eNOS mRNA. The rate of eNOS transfection was 39.6 ± 3.4%. Immunofluorescence staining displayed that subcellular localisation of eNOS was most prominent in plasma membrane and perinuclear regions of the cell. The eNOS activity of eNOS transfected cells had not increased significantly, whereas, in the presence of Ca^{2+}, L-Arg, EDTA, and L-NAME, the eNOS activity was 96.98 ± 13.47, 32.57 ± 6.39, 11.63 ± 3.02, 15.56 ± 7.34 U/ml respectively and the NO level was 55.34 ± 11.19, 9.43 ± 4.51, 2.63 ± 1.41, 3.73 ± 1.65 mmol/L, respectively. Meanwhile, the growth curves of EC shifted. SNP also had obvious growth-inhibiting effects on the cells. Together, the ECV304 growth curve went downward in a NO concentration-dependent manner.

Conclusion: Eukaryotic expression vector pcDNA3.1-eNOS was constructed successfully with the ability to express human eNOS mRNA and protein in EC effectively. The activity of eNOS in EC could be regulated by certain exogenous factors. Ca^{2+} was an important factor promoting NO release and excess NO had a cytotoxic effect on EC in vitro. Controlled release of NO in vivo and polygenic measurements might be considered in more clinical gene therapy studies.

Key words: eNOS; eukaryotic expression vector; endothelial cells; gene therapy

Introduction

Cardiovascular diseases are the leading cause of illness and death in the world. Although many aspects regarding restenosis after vascular operation and angioplasty remains unclear, one strong candidate for controlling vascular restenosis is NO, a potent biological vasodilator produced in vascular endothelium from L-arginine by the endothelial nitric oxide synthase (eNOS). In the near future, molecular medicine will be applied to numerous cardiovascular disorders including

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restenosis. Increasing eNOS expression and NO levels to treat cardiovascular disease by transfecting eNOS in endothelial cells (EC) have shown to be a beneficial therapeutic strategy.

Unfortunately, positive experiments did not always translate to positive trials, inducing doubt and scepticism in both the clinical and scientific communities. Endothelial dysfunction defined as the impaired ability of vascular endothelium plays a key role in the development of restenosis [1]. The beneficial effects of NO on restenosis in the vascular wall include the inhibition of smooth muscle cell (SMC) proliferation. However, the role of NO in regulating EC proliferation is controversial in these studies. Recently it was shown that adenoviral-mediated gene transfer of eNOS to EC affected endothelial cell proliferation [2]. Due to the less serious immunogenic concerns and acute inflammation, non-viral vectors can be used as gene vector in cardiovascular gene therapy in the future. However, developing an efficient gene therapeutic approach also implies designing safe and efficient gene delivery reagents. The track record of liposomal transfection vectors has indeed been encouraging. On the other hand, evaluating the eNOS expression and activity after transfection as well as the effects of exogenous factors that influence eNOS activity might require more trials. In this present study, we constructed the non-viral, eukaryotic expression vector pcDNA3.1-eNOS. We effectively transfected this construct, mediated by cationic liposome (LF2000 Invitrogen) into Human umbilical vein endothelial cells (ECV304) in vitro. The transfection rate, the eNOS activity in EC (in the presence of various chemical factors) and the influence of transfection on EC proliferation were assessed in order to improve the safety and efficiency of this gene therapeutic strategy.

Materials and methods

Construction of pcDNA3.1-eNOS pAdCMV-eNOS was kindly provided by Xin-Yu Wu. With EcoRI digestion, the cDNA encoding human eNOS was excised as a 4.0 kb fragment. The eNOS cDNA was then re-cloned into the EcoRI site of pcDNA3.1(+), between the strong enhancer/promoter of the cytomegalovirus (CMV) immediate early genes and the simian virus (SV) 40 polyadenylation signals. The plasmid also contains a ampicillin and a neomycin resistant gene. The cloning direction was verified by EcoRI and XhoI restriction enzyme digestion. The plasmid map of the constructed pcDNA3.1-eNOS is illustrated in figure 1.

The plasmid DNA used for transfection was purified with a plasmid purification kit (Qiagen) according to the instruction. In brief, 100 ml cultured DH_{5a}· containing plasmid DNA was harvested. After the procedure of resuspension, lysis, and neutralisation, the supernatant containing plasmid DNA was applied to the Qiagen-tip. The DNA was redissolved in a suitable volume of Tris edetic (TE) acid buffer. DNA concentration was determined by UV spectrophotometry.

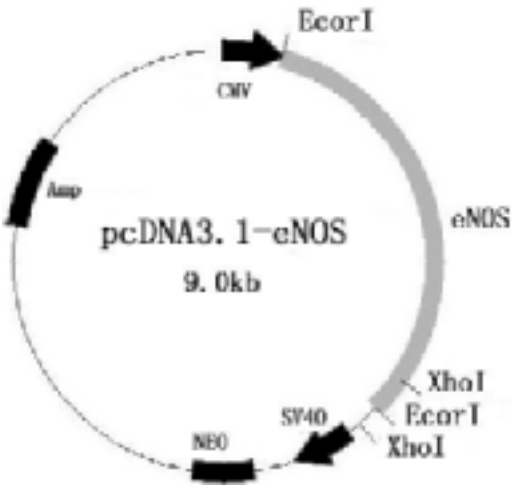


Figure 1

The plasmid map of the constructed pcDNA3.1-eNOS.
CMV: the enhancer/promoter of the cytomegalovirus immediate early genes
eNOS: human endothelial nitric oxide synthase cDNA
SV40: simian virus 40 promoter
NEO: the neomycin resistant gene
Amp: the ampicillin resisitant gene

Cell culture and transfection

The Human umbilical vein endothelial cells (ECV304) were maintained in DMEM medium supplemented with 10% fetal calf serum, benzylpenicillin 100 kU/L, and streptomycin 0.1 g/L, at 37 ºC in a humidified atmosphere containing 5% CO_2. The plasmid DNA (pcDNA3.1-eNOS and pcDNA3.1 as control) was transfected into ECV304 by cationic lipofectamine (LF2000, Invitrogen). The day before transfection, the ECV304 were trypsinised, counted and plated in 24-well plates at 1×10^5 cells per well resulting in 90–95% confluency on the day of transfection. Cells were plated in 0.5 ml of DMEM containing 10% fetal bovine serum (FBS) without antibiotics. For each well of cells to be transfected, 1.0 μg of DNA (concentration I) and 3.0 μl of LF 2000 (1 μg/μl) reagent were diluted respectively into 50 μl of DMEM without serum and antibiotics, and incubated for 5 min at room temperature. Meanwhile, 2.0 and 4.0 μg DNA (concentration II and III) were used as control in an independent experiment for establishing a dose-response curve. Once the LF2000 reagent was diluted, it was combined with DNA within 30 min, and then incubated at room temperature for 20 min to allow DNA-LF2000 reagent complexes to form. The DNA-LF2000 reagent complexes were added directly to each well and mixed gently by rocking the plate back and forth. The final concentration of eNOS DNA in culture fluid (concentration I/II/III) was approximately 1.667/3.334/6.668 μg/ml respectively. The cells were incubated at 37 °C in a CO_2 incubator (5% CO_2) for 48 hours. Subsequently they were assayed for transgenic expression by reverse transcription-polymerase chain reaction (RT-PCR). Non-transfected cells and pcDNA3.1 transfected cells served as control. For the assessment of the transfection rate of DNA-LF2000 reagent complexes, we transfected pcDNA3.0-EGFP and co-transfected pcDNA3.1-eNOS with pcDNA3.0-EGFP respectively mediated by the same lipofectamine. We evaluated the fluorescence expressive cells by fluoroscopy and flow cytometry in the early study.

Identification of eNOS mRNA in transfected cells

Total RNA was isolated from ECV304 using the Fast-Track kit (Invitrogen, San Diego, CA). The resulting pool was amplified by RT-PCR using the primers specific for eNOS and β-actin. For eNOS, the primers were 5'-AGA TCC ACC TCA CTG TAG CTG TGC-3'(sense) and 5'-GTA ACA TCG CCG CAG ACA AAC ATG-3'(antisense). For the quantitation of mRNA, primers were used in a reaction involving one cycle of reverse transcription at 50 °C for 30 min and at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 72 sec. Then the resulting RT-PCR fragments were electrophoresed on 1% agarose gels. The PCR products were 499 bp in length and identified by agarose-gel electrophoresis. For β-actin amplification, the primers were 5'-GGGGTGTTGAAGGTCTCAAA-3' and 5'-GGC-ATCCTCACCCTGAAGTA-3', and the PCR conditions involved denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s for 30 cycles. β-actin products were 202 bp in length used as an internal control.

Immunocytochemistry assessment of eNOS protein expression

Non-transfected cells and transfected cells were trypsinised, washed and resuspended in complete cell culture medium; counted; washed twice with Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl) and sliced by Cytofuge (Cyto-centrifuge System M801–22, StatSpin). Cells were fixed in acetone for 5 min at 20 °C and then rinsed twice with Dulbecco's phosphate-buffered saline (PBS) plus 0.1% bovine serum albumin (w/v) for 5 min at room temperature. The slices were dried at 37 °C, rehydrated and incubated with diluted primary antibody at room temperature for one hour. The antibody, a rabbit monoclonal anti-human eNOS (DAKO, UK) was applied at a 1:50 dilution. The slices were then washed and a secondary antibody, Fluorescein-Conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (DAKO, UK) at a 1:100 dilution was applied for 60 min at 37 °C. After being washed three times for 10 minutes with PBS and the anti-quench agent, the slices were put on coverslips for fluorescence microscopy.

Determination of eNOS Activity and NO release

The enzyme activity of NOS and the NO production in transfected and non-transfected cells were assessed by measuring its breakdown products from each well (number of wells = 8) after 48 hours (Jiancheng Medical Institute, China). Meanwhile, the same was measured in the presence of Ca^{2+}. Furthermore, the enzyme activity and the NO level were studied respectively in the presence of L-arginine (L-Arg, 2×10^{-3} mol/L), Ca^{2+} (2×10^{-3} mol/L), Ethylene-diaminetetra-aceticacid (EDTA, 1×10^{-3} mol/L), and L-NAME (1×10^{-3} mol/L). The agents were added to the culture medium (n = 8) 6 hours after pcDNA3.1-eNOS transfection. The pcDNA3.1 transfected and non-transfected cells (n = 8) served as controls. In all groups the ECV304 cell number and culture medium volume were 106 and 2 ml and each experiment was repeated twice for the measurement accuracy.

MTT and Flow Cytometry assay for EC proliferation

Cell preparation, plating, incubation and transfection were the same as described above. After the culture supernatant was aspirated, 100 ml MTT (5 mg/ml Sigma) stock solution in PBS was added to 8 wells per group, every 12 hours, over 60 hours. After 6 hours of incubation, 100 ml 10% SDS (Sigma) containing serum was added to each well. The plate was mixed gently by rocking back and forth until the blue sedimentation was completely dissolved. Then the absorbances were read by Tecan's sunrise absorbance microplate reader (A-5082) [3]. For additional evaluation of the NO effect on cell proliferation, we set up an isolated exogenous NO donor sodium nitroprusside (SNP) group. SNP (10 micromol/L mol/L) was added to culture medium 6 hours after transfection. For the measurement accuracy, the same experiment was repeated twice. Each experimental group consisted of 8 wells.

For further research an eNOS DNA dose-response curve was established to evaluate EC viabilty, based on 2 DNA concentrations. In addition, the experiments were performed in the presence or absence of Ca^{2+} to investigate this electrolyte's effect on eNOS expression. For the measurement accuracy, experiments were repeated twice. Each experimental group consisted of 8 wells.

The survival and apoptotic rates of ECV304 were determined by propidium iodide (PI) and annexin V staining. Cells were digested by 0.25% trypsin at 37 °C for 20 min and fixed with ice-cold 70% ethanol at a cell density of 1×10^6 ml^{-1}. PI and annexin V were then added and incubated with the cells in the dark for 30 min until detection by flow cytometry [4].

Statistical analysis

The data were expressed as mean ± SD. Statistical comparisons between groups were performed using Student's t test. Differences among means were considered significant at $p < 0.05$. All the data were analysed with the statistical software SPSS10.0.

Results

Identification of constructed eukaryotic expression vector pcDNA3.1-eNOS

Plasmid pcDNA3.1-eNOS was cut into two EcoRI fragments of 5.4 kb and 4.0 kb indicating that the inserted fragment was a monocopy rather than a multicopy. The pcDNA3.1-eNOS and pcDNA3.1-as-eNOS plasmid could be distinguished by digestion with XhoI; the first construct in which eNOS was inserted in the same direction as the CMV promoter, was cut into one larger fragment of 9.0 kb, and the second construct was cut into two fragments of 5.4 kb and 4.0 kb (figure 2).

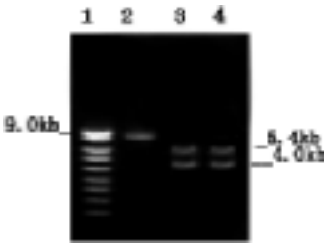


Figure 2

Identification of the constructed pcDNA3.1-eNOS.
Lane 1: DNA marker
Lane 2: pcDNA3.1-eNOS digested by XhoI
Lane 3: pcDNA3.1-eNOS digested by EcoRI
Lane 4: pcDNA3.1-as-eNOS digested by XboI

Transfection rate calculation with cationic lipofectamine

pcDNA3.0-EGFP was transfected into ECV304, mediated by cationic lipofectamine to evaluate the fluorescence expressive cells by fluoroscopy. We measured 38.8 ± 4.4% (n = 12). After co-transfection of pcDNA3.1-eNOS with pcDNA3.0-EGFP mediated by lipofectamine into cells, the cell fluorescence expression rate was 33.4 ± 2.8% (n = 12) and the flow cytometry transfection rate was 39.6 ± 3.4% (n = 12). The two control groups showed no expression in both fluoroscopy and flow cytometry.

eNOS mRNA expression in transfected cells

Total RNA extracted from pcDNA3.1-eNOS and pcDNA3.1 transfected cells were reversely transcribed to check whether eNOS mRNA could be expressed in eNOS gene transfected cells. RT-PCR detection with β-actin primers revealed a 202 bp fragment in cells from both groups. However, RT-PCR detection with eNOS primers revealed a 499 bp fragment only in pcDNA3.1-eNOS transfected cells rather than in pcDNA3.1 transfected cells. No fragments were detected in the negative control (figure 3).

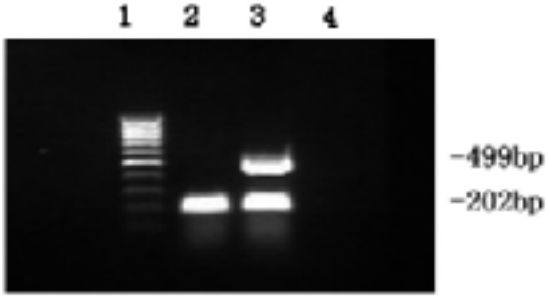


Figure 3

RT-PCR detection of eNOS and β-actin mRNA expression in transfected ECV304
Lane 1: DNA marker
Lane 2: ECV304 transfected with pcDNA3.1
Lane 3: ECV304 transfected with pcDNA3.1-eNOS
Lane 4: negative control

Detection of eNOS protein expression in ECV304

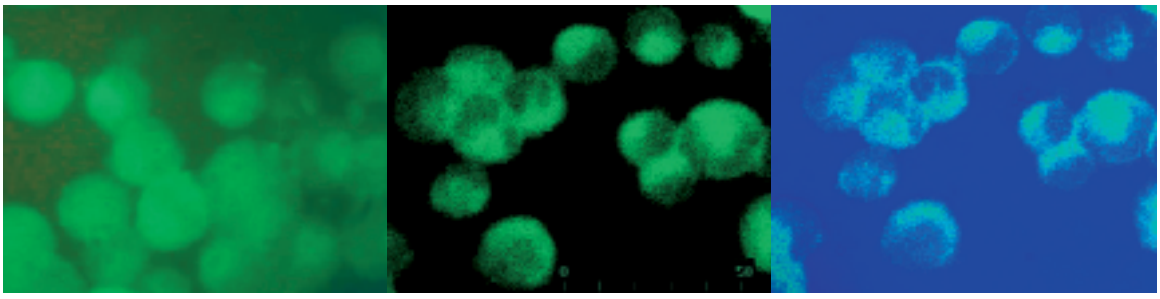
Immunofluorescence staining displayed that the expression of eNOS protein in the ECV304 transfected by pcDNA3.1-eNOS was significantly enhanced. Subcellular localisation of eNOS was assessed by LSCM and 3-D Morphologic Fluorescence intensity indicating that eNOS protein was most prominent in plasma membrane and perinuclear regions of the cell (figure 4 A, B, C).

Determination of eNOS activity and NO level

The basal activity of eNOS in non-transfected cells was within the normal range and it did not significantly differ from pcDNA3.1-eNOS and pcDNA3.1 transfected groups (P >0.05). However, in the presence of Ca^{2+}, activity of NOS and the NO production were increased significantly. In the presence of L-Arg, the activity of eNOS and NO level were similar to the preceding three groups. However, the synthase activity was dramatically decreased in the presence of L-NAME and EDTA; the latter agent was the strongest inhibitor. After the eNOS transfection, calcium promoted the activity of NOS and the NO production up to 4-fold. All of the associated data are shown in table 1.

Figure 4

Immunofluorescence staining of eNOS expression in ECV304 transfected with pcDNA3.1-eNOS (4A). Positive signal located mainly in plasmalemma and perinuclear regions of cells by LSCM in 3-D Morphologic Fluorescence intensity (4B, 4C). 400 ×



A B C

Table 1

The activity of eNOS and NO level after cDNA3.1-eNOS transfection in ECV304 cells ($\bar{x}$ ± SD, n = 8)

Groups	Exogenous factors	NOS (U/ml)[1]	NO (μmol/L)[2]
Controls		21.23 ± 4.67	6.32 ± 2.12
Controls	Ca^{2+} [3]	37.85 ± 7.13*	10.84 ± 2.94*
pcDNA3.1		18.94 ± 3.68	5.14 ± 2.44
pcDNA3.1	Ca^{2+} [4]	41.53 ± 6.34*	9.66 ± 3.13*
pcDNA3.1-eNOS		30.32 ± 5.63	8.23 ± 4.12
pcDNA3.1-eNOS	L-Arg[5]	32.57 ± 6.39	9.43 ± 4.51
pcDNA3.1-eNOS	L-NAME[6]	15.56 ± 7.34*	3.73 ± 1.65*
pcDNA3.1-eNOS	Ca^{2+}	96.98 ± 13.47*	55.34 ± 11.19*
pcDNA3.1-eNOS	EDTA[7]	11.63 ± 3.02*	2.63 ± 1.4*

* compared with control, pcDNA3.1, L-Arg and pcDNA3.1.0-eNOS groups, P <0.05
[1] NOS: nitric oxide synthase, [2] NO: nitric oxide, [3] Ca^{2+}: calcium, [4] eNOS: endothelial nitric oxide synthase,
[5] L-Arg: L-arginine, [6] L-NAME: N-nitro-L-arginine methylester, [7] EDTA: Ethylene Diamine Tetraacetic Acid

Evaluation eNOS transfection on proliferation of ECV304

MTT assay indicated that the ECV304 proliferative curves were similar in pcDNA3.1, EDTA and non-transfected groups. However, the cell proliferation in the pcDNA3.1-eNOS transfected group was inhibited to some extent and was inhibited strongly in the presence of calcium. Apoptosis in these two groups was elevated compared to the three groups as described above. In the SNP group, ECV304 were notably suppressed within 12 hours, and cell death occurred in 36 hours. In flow cytometry analysis, SNP promoted apoptosis (25.53 ± 1.36% in 12 hours and 71.85 ± 6.81% in 24 hours). Together, the ECV304 growth curve went downward in a NO concentration-dependent manner. All of the different growth curves of EC are shown in figure 5.

eNOS transfection caused a downward shift in the growth curve of EC proliferation in the presence of Ca^{2+}. Moreover, this effect seemed eNOS transfection-dose-dependent. There were significant differences between control and transfected groups, but no differences among transfected groups. However, without any exogenous influencing factors, there were no significant differences between the transfected groups including controls (figure 6, 7).

Figure 5

The ECV304 growth curves changed in a NO concentration-dependent manner. Results from non-transfected cells, pcDNA3.1 and pcDNA3.1-eNOS transfected cells in the presence or absence of Ca^{2+}, EDTA and isolated SNP respectively. The data are expressed as mean absorbence by spectrophotometry (n = 8).
MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for the proliferation of surviving cells, which is directly proportional to the level of the formazan product created
MTT(OD): MTT optical density
EDTA: Ethylene Diamine Tetraacetic Acid
SNP: sodium nitroprusside
eNOS: endothelial nitric oxide synthase

* Significantly different from control group ($P < 0.05$)

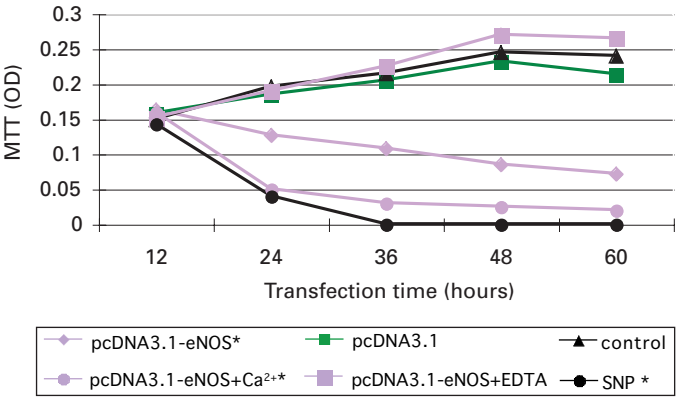


Figure 6

The ECV304 growth curves in a transfected eNOS concentration dependent manner: Concentration I, II, III and the control group concomitant with the Ca^{2+} respectively. The data are expressed as mean absorbence by spectrophotometry (n = 8).
MTT(OD): MTT optical density
eNOS: endothelial nitric oxide synthase
EC: endothelial cells
Concentration I/Concentration II/ Concentration III: 1.667/3.334/6.668 μg/ml of eNOS DNA transfected respectively
Ca^{2+}: concentration of Ca^{2+} is 2×10^{-3}mol/L

* No significantly difference among all of transfected and control groups ($P > 0.05$)

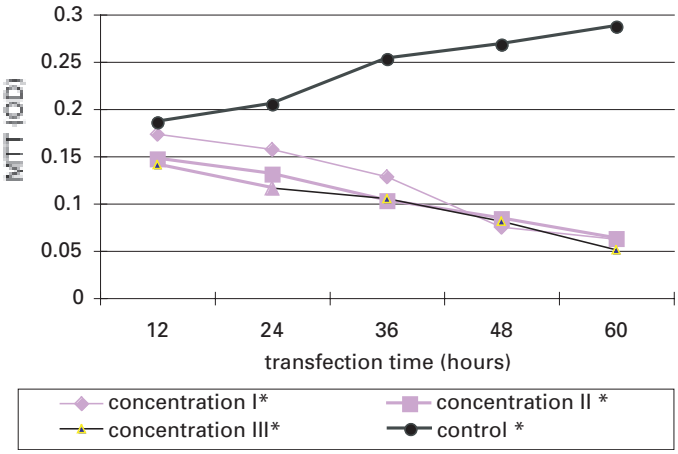
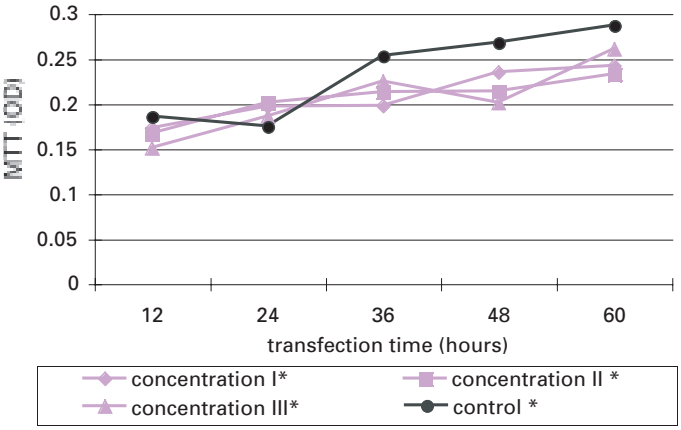


Figure 7

The ECV304 growth curves in a transfected eNOS concentration dependent manner: Concentration I, II, III and control group without any exogenous influencing factors.
The data are expressed as mean absorbence by spectrophotometry (n = 8).
MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for the proliferation of surviving cells, which is directly proportional to the level of the formazan product created
MTT(OD): MTT optical density
eNOS: endothelial nitric oxide synthase
EC: endothelial cells.
Concentration I/Concentration II/ Concentration III: 1.667/3.334/6.668 μg/ml of eNOS DNA transfected respectively

* No significantly difference among all of transfected and control groups ($P > 0.05$)



Discussion

Endothelium-derived NO, produced by a constitutive low output eNOS, which is found in the endothelial cells (hence the "e") that line the lumen of blood vessels, was a key molecule in the regulation of the vascular tone and homeostasis [5–9]. The EC and eNOS were damaged in injured arteries. Transfection of the eNOS gene into cells of the vascular wall was sufficient to recover the NO production. However, how to increase the eNOS transfection rate and how to regulate the activity of eNOS remained a problem. Mean-while, the re-endothelialisation proved to be the more important process in blood vessel repair [10] and the NO effect on EC survival was questioned [2, 11].

Transfection vectors commonly used in gene therapy are mainly of two types – viral and non-viral. They have been experimentally applied in eNOS gene transfer to cardiovascular systems [12–14]. Efficiencies of viral transfection vectors were unquestionably superior to their non-viral counterparts. However, due to the less serious immunogenic concerns and acute inflammation risk, non-viral vectors are a more probable choice in cardiovascular gene therapy in the future [15]. Liposomes, microscopic bubbles of fatty molecules (lipids) surrounding a watery interior, have long been viewed as non-viral gene vector delivery systems for their similarity to cell membranes [16]. In our study, the eNOS cDNA was subcloned into the pcDNA3.1 eukaryotic expression vector containing an enhanced express promoter and was transfected into ECV304 mediated by cationic lipofectamine. The rate of expression was demonstrated by RT-PCR and immunofluorescence staining. eNOS is a peripheral membrane protein that targets specific intracellular domains, including the Golgi and cholesterol, and sphingolipid-rich microdomains of the plasma membrane, ie caveolae, which accorded with images from the LSCM in our study. Based on our findings, the proper vectors and improved transfection techniques can ensure the clinical success of non-viral gene therapy.

Different from iNOS, eNOS is a calcium/calmodulin dependent enzyme that is mainly located in EC. eNOS is activated by Ca^{2+} and produces physiological amounts of NO (normally 90% of circulating NO is derived from this enzyme) in a physiological environment and might be enhanced by certain agents [17]. Nitric oxide is a highly reactive gas with a short half-life of approximately 6–30 s and NOS is the dominating limiting factor for NO production [18, 19]. Therefore, the determination of NOS activity is an important part in the study on NO function. In our study, exogenous eNOS was expressed well as demonstrated in immunohistochemical staining of ECV304. However, the enzyme activity was still similar to that of the control groups. On the other hand, the enzyme activity was elevated markedly in the presence of calcium but decreased in the presence of specific eNOS inhibitors ie L-NAME and calcium conjugation EDTA. In vitro, simulative physiological research suggested that the stable eNOS had been modulated by at least two factors, the shearing forces acting on the luminal surface of vascular endothelium and increased flow velocity [20]. Similarly, it seemed that after the successful transfection and expression, eNOS dysfunction must be activated by some necessary factors. We considered that multiple physiological factors regulated calcium release and eNOS activity in vivo and that at least calcium was one of the final necessary ingredients in maintaining NO release.

The role of L-Arg, as the specific substrate of eNOS, was surprising in this study. Logically, the straightforward approach to increase NO production was to provide additional substrate to the eNOS [21]. Early studies had shown that increasing exogenous levels of arginine by local and systemic administration of L-arginine, a precursor of NO in humans, inhibited restenosis in rabbits by increasing NO production in the injured artery [22]. Providing supplementary substrate to individuals with inadequate NO is proposed to increase NO production by the endothelium. This therapeutic paradigm has met some success as well as uncertain results in clinical studies [23–25]. According to our results, the intracellular levels of L-Arg were in the millimolar range, whereas the enzyme's K_m for substrate was in the micromolar range [26]. Supplementary L-arginine increases culture medium levels of the amino acid, but due to the great difference between substrate concentration and K_m, substrate availability was unlikely to be rate limiting even in the presence of arginase. For clinical benefits the possible actions of L-arginine might require more trials.

Some recent literature reported that SMC transfected with eNOS in vitro increased co-cultured EC migration and capillary tube formation. Moreover, EC transfected with eNOS synthesise an increased amount of NO, and simultaneously, just like in our study, this increase is inhibited by L-NAME [27, 28]. Our data demonstrated for the first time that the NO levels were similar in eNOS transfected and non-transfected EC. In addition, the dose-dependent transfection experiment revealed that the eNOS transfection did not affect significantly the EC viability in the absnece of exogenous factors. Only in the presence of Ca^{2+} the eNOS transfection increased NO release significantly compared to unstimulated transfected EC or in the presence of L-Arg. It was very different from previous experimental results [28]. The reason of this phenomenon was not clear and will be studied in our next step. However, at least Ca^{2+} has shown to be an important patho-physiological mediator of eNOS activity.

On the other hand, eNOS transfection usually promotes the EC proliferation and migration. However in our study, transfected EC proliferation was inhibited compared to the controls in the first 12 hours and the effect became obvious in the presence of calcium. Moreover, apoptosis occurred within

48 hours in the presence of SNP. It showed that EC are very sensitive to the NO cytotoxic effects in vitro. Although the study demonstrated that eNOS transfer is a useful tool for the study of targeted genes in vascular biology [29], the subject remains controversial. Recent studies showed that excess NO as an oxidant can cause lipid peroxidation, cellular dysfunction and apoptosis or death. However, the cascade of NO-mediated apoptosis was not fully understood [30–32]. Our experiments suggest that the NO effect on EC in vivo and its suitable therapeutic concentration for endothelial function require more trials, for many aspects regarding exogenous factors affecting EC proliferation remained unclear. In summary, our study results support a rare phenomenon that relatively high concentrations of NO seemed to produce a cytotoxic effect on EC. This suggests that the cell-based eNOS gene transfer may be a careful approach to increase new blood vessel formation in vivo.

Based on our study, we suggest that more attention must be paid to the side effects of NOS transfection on EC in clinical gene therapy. Mechanisms regulating the release of NO in vivo and proper coadjusment of polygenic combination measurements are recommended subjects for future correlated research.

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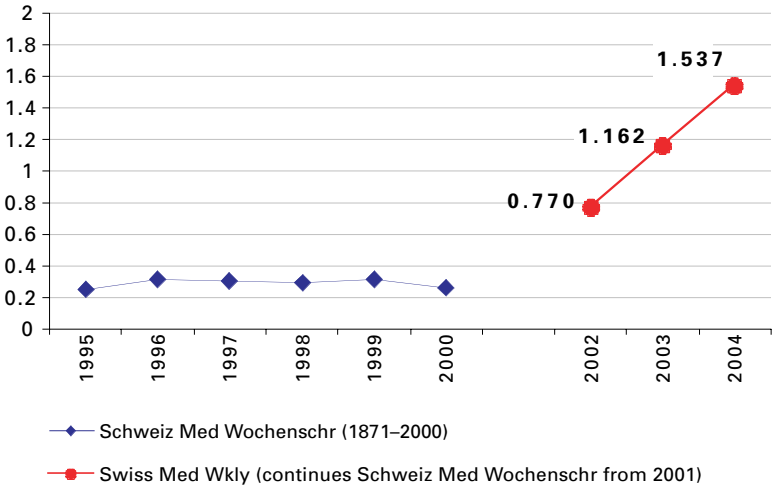

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