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Differential Ability of Human Endothelial Cells to Internalize and Express Exogenous DNA

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Summary. **Vascular endothelium gene expression regulates blood-vessel wall interactions, vascular permeability, smooth muscle cell growth and tone. The possibility to introduce exogenous DNA or RNA sequences in endothelial cells represents a novel therapeutic approach of vascular disease. The aim of the work was to investigate the ability of endothelial cells to internalize and express exogenous DNA sequences. Human umbilical vein endothelial cells (HUVEC) were transfected with either a 780 bp fluorescein-labeled DNA (FITC-DNA) or pEGFP-C1 plasmid encoding for a green fluorescent protein (GFP), using the cationic liposome DOTAP as transfection reagent. The transfected cell population was passed through a FACScan apparatus and percentage of fluorescent cells was determined using a FACScan analysis programme. The SW620 tumor-derived cell line was used as control. The percentage of FITC-DNA positive cells was 66.0% for HUVEC and 45.0% for SW620 cells. On the contrary, the percentage of GFP-positive cells was 13.8% and 43% for HUVEC and SW620, respectively. By increasing the amount of DNA as well as the protocol of administration the percentage of GFP-positive HUVEC was enhanced suggesting a rapid degradation of DNA in the HUVEC cytoplasm.**

Key Words. **human endothelial cells, pharmacology, gene delivery, gene expression**

1. Introduction

Vascular endothelium regulates blood-vessel wall homeostasis through the production of factors regulating vessel tone, coagulation state, cell growth, cell death, and leukocyte trafficking [1]. Endothelial cell dysfunction is involved in a wide spectrum of vascular disorders including inflammation, hypertension, atherosclerosis and transplant rejection [2–4]. Although significant progress has been made in treating vascular disorders, novel therapeutic approaches that effectively prevent, arrest, or reverse endothelial dysfunction are actually under study. Among these, the possibility of introducing exogenous DNA or RNA sequences in endothelial cells is particularly attractive in terms of potential therapeutic applications [5]. Several viral and non-viral transfection methods have been tested to transfer nucleic acids to living cells. Among non-viral methods, cationic liposomes seem to be the most promising [6,7]. Moreover, the low risk of intrinsic toxicity or in-vivo pathogenicity renders cationic liposomes attractive for research aimed toward vascular gene therapy [8–12]. The present work was aimed at better defining the potential of endothelial cells to internalize and express exogenous DNA sequences.

2. Methods

2.1 Cell cultures

Human umbilical vein endothelial cells (HUVEC) were grown on gelatin-coated dishes containing Medium 199 (25 mM HEPES) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine (all from Gibco BRL), 80μg/ml heparin (Sigma Chemical Company) and 20 μg/ml endothelial cell growth factor (Roche). All experiments were performed with cells at passage 5–8. Adenocarcinoma SW620 cell line was routinely grown in Dulbecco's Modified Eagle medium (DMEM), containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin. Cell cultures were maintained at 37°C in a humidified incubator with 6% CO_2.

2.2 FITC-DNA

The 780 bp fluorescein-labeled DNA (FITC-DNA) was obtained by Polymerase Chain Reaction (PCR) of a plasmid sequence using one of the primers conjugated to fluorescein. The primers were synthetized by routine protocols using the automatic Expedite 8900 DNA Synthetizer (PerSeptive Biosystem) and the standard phosphoramidite chemistry. The aminomodifier C_6 (Glen Research) was inserted at the 5′ end of the reverse primer by on-line conventional coupling step. Oligonucleotides were fully deprotected by standard concentrated ammonia treatment, and purified by ion exchange preparative liquid chromatography as

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already described [13]. To label the amino-derivatized oligonucleotide, the crude product was reacted for up to 20 h in the dark at room temperature with fluorescein isothiocyanate, (FITC, Pierce), in 250 mM bicarbonate buffer at pH 9.0, according to manufacturer's instructions. At the end, oligonucleotide was ethanol precipitated, further purified in order to separate labeled and unlabeled material using a 7.8×300 mm μBondapak C18 reverse-phase column equilibrated at 4 ml/min with 0.1 M ammonium acetate pH 6.5, and run under a linear 10–40% acetonitrile gradient in 20 min. Fluorescent samples were desalted and stored in the dark at -20°C. The PCR reaction was performed in a volume of 50 μl containing 100 ng of DNA template, 0.2 mM dNTPs, 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase, and 10 μM of both forward and reverse primers. The PCR conditions were: denaturation at 94°C for 2 min for the first cycle and 1 min for 30 cycles, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The 780 bp PCR fragment was extracted from 1.5% agarose gel, ethanol-precipitated, resuspended in water and then used to transfect cells.

2.3 Plasmid DNA

The pEGFP-C1 plasmid (Clontech) is 4.7 kb long DNA sequence and contains the cytomegalovirus promoter. The plasmid carriers a humanized sequence coding for a mutated *Aequora victoria* green fluorescent protein (GFP) which becomes fluorescent after synthesis and folding. pEGFP-C1 was amplified in Escherichia coli by a standard procedure [14] and isolated using a Concert^{TM} DNA purification kit (Gibco BRL).

2.4 Transfection procedure

HUVEC and SW620 cells were seeded at 1.25×10^5 cells per 30 mm diameter dish the day before transfection in order to have mitotically active cells [15]. DNA and the cationic liposome DOTAP (Roche) were diluted in HEPES 20 mM, pH 7.4, according to manufacturer's instructions. Aliquots of DNA, corresponding to 15 pg/cell, were added to DOTAP (DNA/DOTAP 1:2 w/w ratio) and the mixture incubated for 10–15 minutes at room temperature. Cells were washed twice in OPTI-MEM (Gibco BRL) and then exposed to the transfection mixture for 8 h, a length sufficient to internalize exogenous DNA [16] without affecting cell viability. Thereafter, cells were washed once with glycine 0.2 M, pH 2.8, to remove any remaining fluorescent extracellular complex [17]. When specified, cells were exposed to 25 μM and 50 μM chloroquine 30 min before and during the transfection. The internalization of FITC-DNA sequence was verified at the end of 8 h transfection whereas that of pEGFP-C1 48 h later to allow GFP protein expression.

2.5 Flow-cytometry

The detection and count of cells containing FITC-DNA or expressing GFP were performed by flow-cytometry on a fluorescence-activated cell sorting apparatus (FACScan, Becton Dickinson). Briefly, transfected cells were harvested by trypsinization, centrifuged at 2000 r.p.m. for 5 minutes and resuspended in 100 μl of PBS. Fluorescence of 5,000–10,000 individual cells was measured. The percent of labeled cells was calculated by subtracting the fluorescence of control cells. The resulting value was considered a measure of the transfection efficiency per viable cells since an appreciable amount of floating cells at the end of transfection of reduction of cell number during the GFP protein expression time was never observed.

3. Results

The expression of a transfected DNA sequence needs first the internalization of the DNA into the cytoplasm and then the translocation into the nucleus. We followed these steps by transfecting HUVEC with DNA/DOTAP complexes containing sequence at either cytoplasmic and nuclear (FITC-DNA) or nuclear (pEGFP) localization. Representative distribution profiles obtained by flow cytometry of transfected populations are shown in Figure 1. 66% $\pm$ 12.6% (mean $\pm$ SD) of HUVEC internalized FITC-DNA and 13.8% $\pm$ 1.1% expressed GFP (Fig. 2).

To evaluate if other cell lines shared the discrepancy, we transfected SW620 cell line. The growth properties of SW620 largely differ from those of HUVEC because SW620 which are tumor-derived cells, continuously divide, and grow without contact inhibition. As anticipated from distribution profiles (Fig. 3), 45% $\pm$ 8.3% of cells internalized FITC-DNA and 43% $\pm$ 10.1% of cells were GFP-positive (Fig. 4). Therefore, it seems that the translocation to the nucleus rather than the internalization of coding DNA waa defective in HUVEC.

Since comparable pEGFP amount was transfected in both cell types, it seems that HUVEC degraded the transfected DNA before the translocation in the nucleus. To investigate whether the limiting step of expression of plasmid sequence in HUVEC was due to DNA amount, we increased the plasmid concentration per cell. The results are shown in Figure 5. We observed that by increasing the plasmid amount from 15 pg/cell (column A) to 30 pg/cell (column B) the percentage of GFP positive cells raised from 13.8% to 29.9%. The concentration of 60 pg DNA/cell was also tested (column C) and 57% of surviving cells were GFP positive. Nevertheless, the transfection complex was toxic in that most of cells died. Therefore, the experiments aimed to ascertain whether chloroquine would enhance the percentage of GFP positive cells were done using 30 pg DNA per cell. The results of exposition of HUVEC to both chloroquine and pEGFP/DOTAP transfection complex are shown in Figure 6. In presence of 25 μM chloroquine the percentage of GFP positive cells significantly increased from 29.9% (column A) to 46.0% (column B)

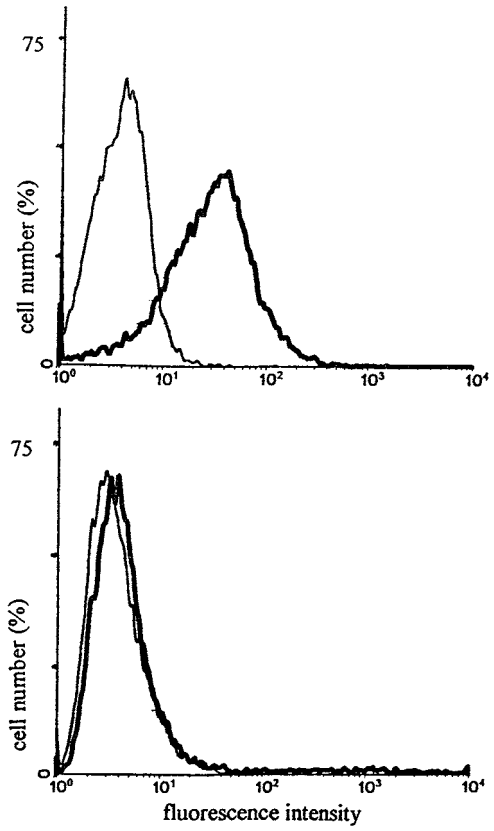

Fig. 1. *Distribution of relative fluorescence intensity versus number of cells following 8 h exposure of HUVEC to FITC-DNA/DOTAP (top) and pEGFP/DOTAP complex (bottom). Thin line, 15 pg DNA; thick line, DNA/DOTAP 1:2 w/w ratio.*

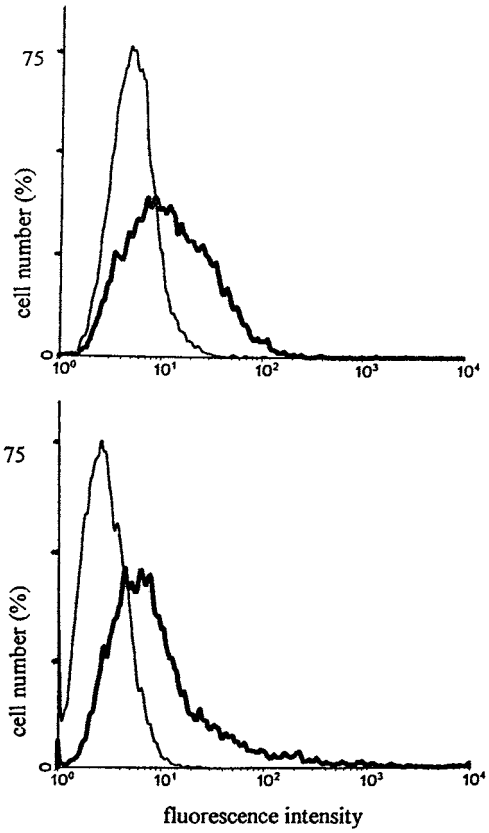

Fig. 3. *Distribution of relative fluorescence intensity versus number of cells following 8 h exposure of SW620 to FITC-DNA/DOTAP (top) and pEGFP/DOTAP complex (bottom). Thin line, 15 pg DNA; thick line, DNA/DOTAP 1:2 w/w ratio.*

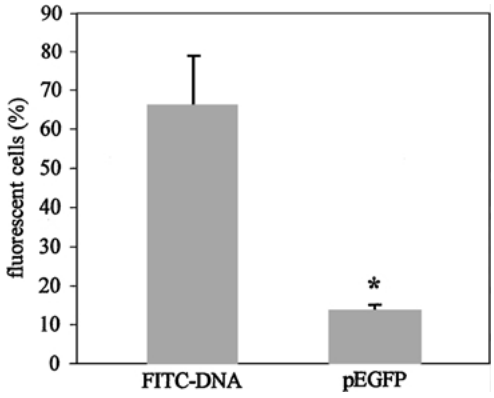

Fig. 2. *Percentage of fluorescent HUVEC exposed to FITC-DNA/DOTAP and pEGFP/DOTAP complex. Each value represents the mean ±SD of 3 different experiments * $p < 0.01$.*

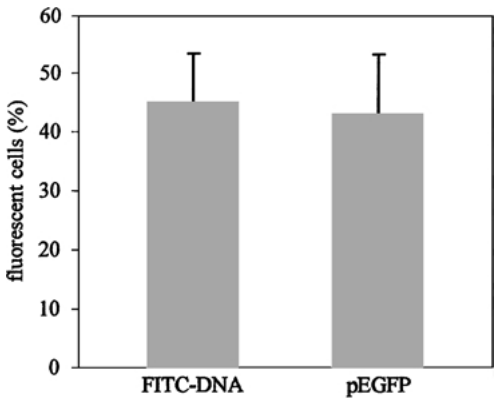

Fig. 4. *Percentage of fluorescent SW620 exposed to FITC-DNA/DOTAP and pEGFP/DOTAP complex. Each value represents the mean ± SD of 3 different experiments. * $p < 0.01$.*

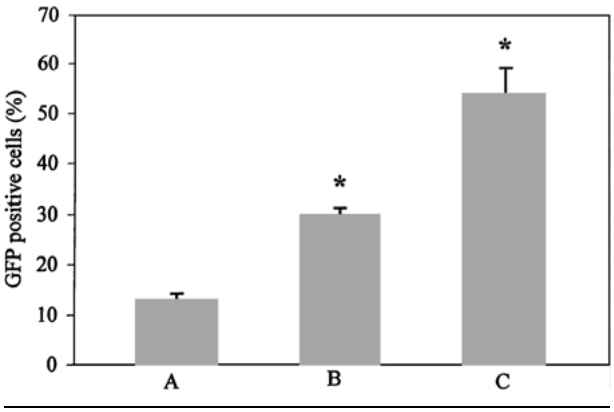

Fig. 5. *Percentage of GFP positive HUVEC following transfection of pEGFP plasmid with DOTAP. A: 15 pg DNA; B: 30 pg DNA; C: 60 pg DNA.* $^{*}p < 0.01$.

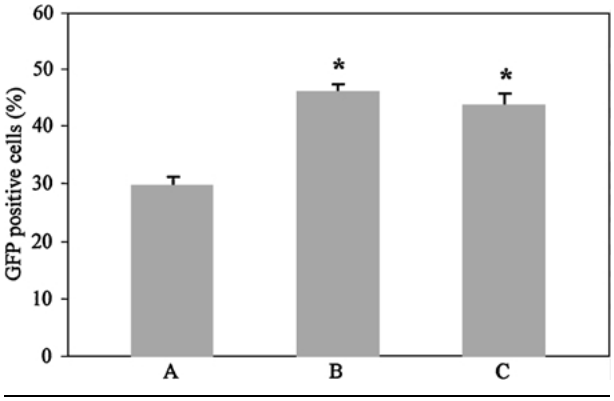

Fig. 6. *Percentage of GFP positive HUVEC following transfection of pEGFP plasmid with DOTAP. A: 30 pg DNA; chloroquine 25* μ*M plus 30 pg DNA; B: chloroquine 50* μ*M plus 30 pg DNA.* $^{*}p < 0.01$.

without any apparent effect on survival. Conversely, the treatment with 50 μM chloroquine (column C) did not further increased the percentage of GFP positive cells.

4. Discussion

Endothelial cells represent an important target for vascular gene therapy. In this work we compared the liposome-mediated delivery of either a non-coding (FITC-DNA) or coding (pEGFP) DNA sequences into primary human endothelial cells. The mechanism by which cationic liposomes mediate the transfection is based on the property they have to absorb the negative charges of tested DNA sequence. The DNA-liposome complex formed is stable and its overall positive charge facilitates the association with the negatively charged cell surface. Therefore the DNA-liposome complex is adsorbed to the cell membrane leading to a transient destabilization of the cell membrane favouring the DNA delivery to the cytoplasm [18,19]. We observed that the uptake of the complex FITC-DNA/DOTAP by HUVEC cells was very efficient. As the acid washing procedure with glycine 0.2 M, pH 2.8 at the end of the transfection is considered appropriate in removing extracellular or membrane-bound fluorescent molecules, a true internalization of this complex in the cytoplasm seems likely. We did not investigate this aspect since it was out of the aim of the work. However, since we have already observed that a pretreatment of HUVEC with dextran sulfate, a donor of negative charges, reduced the FITC-DNA uptake by 60% (unpublished results), it is highly probable that the candidate molecules that mediate the delivery throughout the cell membrane might be proteoglycans [20] which are particularly abundant in endothelial cells [21] and strongly affect the frequency of gene transfer [22,23].

The data obtained by transfecting pEGFP plasmid gave further information. The percentage of HUVEC expressing GFP was 13.8%. This value, which is higher than 0.68% reported for endothelial cells [24] and 10% reported for ECV304, an immortalized endothelial cell line of human umbelical vein origin [25], demonstrates that the protocol of transfection was effective. A rigorous schedule of culturing before transfection, the passage number of cells in culture, DNA amount to be transfected and the amount of the transfectant, the cell density are variables that affect the transfection efficiency. In our experience, HUVEC grow very well up to the 4th passage in culture ant then start to degenerate. HUVEC used in this work were indeed very well growing cells. Probably this is relevant in determining the high transformation efficiency. Nevertheless, there are considerable differences between HUVEC and endothelial cells obtained from arteries of adult patient. Therefore, we caution that this could represent a potential limitation of our findings. It is notewhorthy that the rates of expression of pEGFP in HUVEC and SW620 cells (13.8% vs 43.3%) differed. It has been reported that primary human cells, similarly to HUVEC, are poorly transfectable. A possible explanation is that primary human cells degrade exogenous DNA at the cytoplasmatic level [26,27]. Lysosomes seem to be responsible for that since primary human fibroblasts transfected with a complex containing lysosomal cysteine protease inhibitors, such as chloroquine, approached transfection efficiency close to that of viral vectors [28]. The proposed mechanism was that the protease inhibitors prevented the maturation of lysosomal nucleases and thereby the degradation of DNA. This implies that increasing amounts of DNA should increase the transfection efficiency. Recent data have shown that the transfection of a coding sequence in endothelial cells reached 20% of cells by increasing the amount of transfected coding sequence [29]. We found indeed (Fig. 5) a two-fold increase of GFP positive cells by increasing the plasmid concentration up to 30 pg DNA per cell and a three-fold increase associating chloroquine

and transfection treatments. These results are in agreement with those of other authors, which indicate that chloroquine analogues favour the release of molecules from the transfection complex to cytoplasm [28]. Treatment with chloroquine increased plasmid expression by improving the efficiency of pEGFP nuclear translocation in HUVEC. Based on this result we suggest that degradation of exogenous DNA in the cytoplasm is responsible for the reduced nuclear translocation of pEGFP in HUVEC. The observation in this study is that the percentage of fluorescent cells with DNA amount would reasonably exclude a reduced translation, but rather a protection of pEGFP from degradation, as responsible for the reduced expression.

Therefore we conclude that endothelial cells were able to internalize exogenous DNA very efficiently and that the reduced expression of coding sequence can be significantly increased by enhancing the concentration of tested plasmid and the protocol of transfection.

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