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Transferrin-Polyethylenimine Conjugate, FuGENE6 and TransIT-LT as Nonviral Vectors for Gene Transfer to the Corneal Endothelium

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Purpose: To investigate the efficacy and pathogenicity of three commercially available nonviral DNA vectors for gene transfer to the corneal endothelium.

Methods: Corneas obtained from New Zealand White rabbits were cultured ex vivo. For cell culture, the corneal endothelial cells were removed and cultured in vitro under standard conditions. Using the vectors, culture cells or ex vivo corneas were transfected with plasmid DNA coding for green fluorescent protein (GFP). Expression of the transduced gene was monitored by fluorescence microscopy. Transfection efficiency was estimated as the percentage of GFP-positive cells identified. The viability and morphology of the endothelium were also examined.

Results: Transferrin-polyethylenimine conjugate was effective in vitro but not ex vivo. FuGENE6 and TransIT-LT mediated the transfer of GFP gene both in in vitro and in ex vivo culture. Their efficiency estimated at day 3 was 28.8% and 38.8% in vitro, and 8.1% and 8.8% ex vivo, respectively. Viability staining revealed no dead cells. Morphological study showed no apparent alteration.

Conclusions: FuGENE6 and TransIT-LT are safe, simple to use, and may be useful alternative methods for gene transfer to the corneal endothelium, avoiding certain side effects of viral vectors. As the efficiency could be enhanced, these nonviral vectors may be promising for practical application. **Jpn J Ophthalmol 2002;46:140–146** © 2002 Japanese Ophthalmological Society

Key Words: Corneal endothelium, gene transfer, green fluorescence protein, nonviral DNA vector.

Introduction

Corneal transplantation is the most successful tissue transplantation procedure in humans. As corneal transplantation increases, more and more donor corneas are required. However, a number of donor corneas available at Eye Banks are deemed unsuitable for transplantation due to unacceptably low endothelial cell count. Adult human corneal endothelial cells (CECs) maintained in situ cannot proliferate spontaneously in organ culture. Although various tissue growth factors have been used to stimulate the native endothelial cell population to proliferate, their efficacy was very limited.[1,2] CECs are also the most important targets in immune response, as endothelial rejection is the most serious type of rejection leading to corneal graft failure. A strategy to transfer a specific gene to donor corneal endothelium prior to transplantation in order to induce mitotic division and achieve desirable cell density or to modulate immune response is a promising approach. A key to this approach is the development of appropriate DNA vectors.

Viral vectors are natural gene-delivery systems, of which adenovirus is the most effective and frequently used for gene transfer to the corneal endothelium.[3,4] However, the potential problems involved with the viral vectors are time-consuming construction, local inflammatory response, limited size of DNA, as well as safety and immunogenicity concerns.[3–7] Although

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a variety of nonviral transfection methods such as membrane integrins, polyamidoamine dendrimers, and electric pulse were tested for transfection of corneal endothelium,[8–10] they were of limited use due to inconvenience or low efficiency. Recently, some nonviral DNA vectors became commercially available and were successfully used for gene transfer into a variety of cells. In this study, we examined the efficacy and pathogenicity of three available nonviral vectors: Transferrin-polyethylenimine (Tf-PEI) conjugate (DuoFect), FuGENE6, a novel blend of lipids (nonliposomal formulation), and TransIT-LT, a liposome of low toxicity, for gene transfer to the corneal endothelium in cultures in vitro and ex vivo.

Materials and Methods

Corneal Specimens

All experiments involving animals reported in this study conform to the ARVO Resolution on the Use of Animals in Research. Corneas were obtained from New Zealand White rabbits weighing approximately 3 kg. Following animal death by intravenous pentobarbital injection, corneas with scleral rim were removed and placed in phosphate-buffered saline (PBS). For ex vivo study, just before transfection, corneas were bisected, placed (endothelial cell surface uppermost) into individual wells of 24-well plates with culture medium, Dulbecco's modified eagle's medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), and 10% fetal calf serum (FCS).

Rabbit CECs Culture

Endothelium with underlying Descemet's membrane was peeled off with jewelers' forceps. The CECs were released into 0.2% trypsin/EDTA solution (Gibco) by shaking at 37°C, then harvested and cultured under standard conditions for ex vivo corneas. For visualizing green fluorescent protein (GFP)-expressing cells by fluorescence microscopy, CECs were grown on sterile glass coverslips placed in 35-mm culture dishes. At the time of transfection, the cultured cells were 50–80% confluent, and only the cells at passage 1–3 were used for in vitro study.

DNA Vector-reagents

All three vectors were purchased from manufacturers: Tf-PEI conjugate (DuoFect), a new method for Tf receptor-mediated PEI-enhanced transfection of eukaryotic cells (Quantum Biotechnologies, Montreal, Canada); FuGENE6, a novel blend of lipid (nonliposomal formulation) (Roche Molecular Biochemicals, Mannheim, Germany); and TransIT-LT, a liposome of low toxicity (Mirus Corporation, Madison, WI, USA).

Reporter Gene

Plasmid pEGFP-C1 (4.7 Kb) was purchased from Clontech Laboratories (Palo Alto, CA, USA). It carries the gene coding for enhanced green fluorescent protein (GFP) under human cytomegalovirus promoter. The plasmid was propagated in *Escherichia coli* DH5 and prepared from overnight cultures by alkaline lysis followed by column purification (Qiagen, Hilden, Germany).

Transfection Conditions

Vector/plasmid DNA complex formation was carried out according to the protocols supplied by the manufacturers. In brief, the vector-reagent and plasmid DNA were mixed in serum-free medium at room temperature before adding to the cultures in fresh medium with or without 10% FCS. Transfected cultures were further incubated in 5% CO_2 at 37°C. Mock-control was performed using a GFP reporter gene in the absence of the vector-reagents.

Fluorescence Microscopy

Following incubation in 5% CO_2 at 37°C, the cultures were washed twice with PBS, fixed in 4% paraformaldehyde for 30 minutes at room temperature, and washed twice in PBS. Expression of GFP fluorescence of in vitro cell culture was monitored by fluorescence microscopy (Axioplan 2, Carls-Zeiss, Oberkochien, Germany). For ex vivo corneas, we used a confocal laser scanning microscope (Laser Scanning Confocal Imaging System, Bio-Rad, Hemel Hempstead, UK) with fluorescein isothiocyanate filter to detect GFP expression. The GFP-positive cells were counted and transfection efficiency was estimated as the percentage of the GFP-positive endothelial cells present per total cells in a microscopic field (provided endothelial cell density in ex vivo cornea is approximately 2000/mm^2). The efficiency of each vector was the result of compilation from three independent experiments using three separate microscopic fields and determined by the *t*-test or Welch's test.

Endothelium Viability Examination

Transfected corneal specimens were exposed to a 0.3% aqueous solution of Trypan Blue for 3 minutes

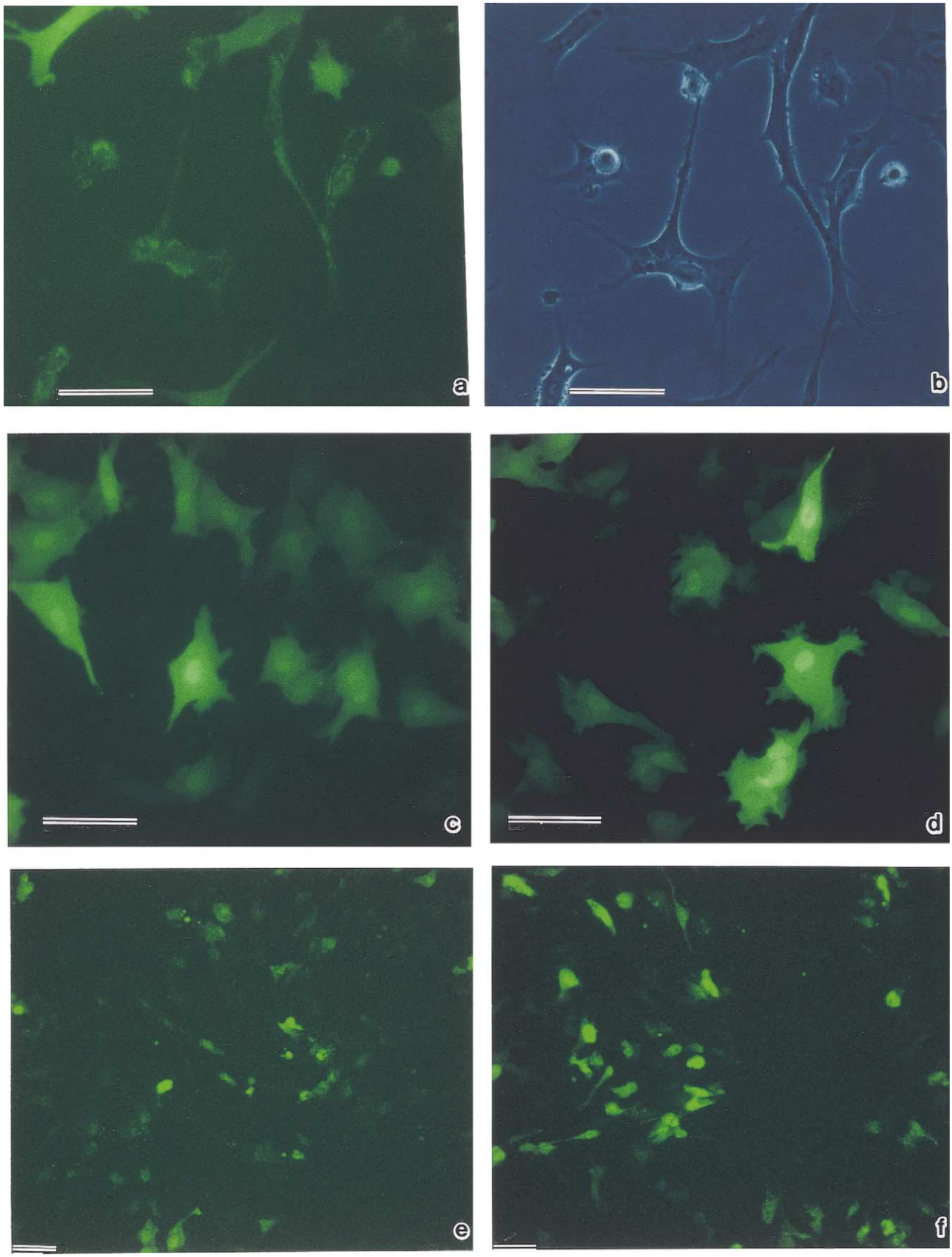

Figure 1. Expression of green fluorescent protein (GFP) reporter gene following transfer to the corneal endothelium using three nonviral DNA vectors. (**a**) Transferrin-polyethylenimine (PEI) conjugate (DuoFect): Fluorescent image shows GFP expression in vitro. (**b**) The same microscopic field in phase-contrast shows low density and elongated form of corneal endothelial cells. (**c**) FuGENE6 (novel blend of lipids) in vitro. (**d**) TransIT-LT (liposome of low toxicity) in vitro. (**e**) FuGENE6 in ex vivo culture cornea. (**f**) TransIT-LT in ex vivo culture cornea. Bars = 60 μm.

and then a solution containing 0.5% Alizarin Red for a further 3 minutes.[11] They were then examined by light microscopy.

Endothelium Morphology Examination

Transfected corneas were prepared by standard techniques[12] and evaluated with a Hitachi S-800 microscope (Hitachi, Tokyo).

Results

In the negative control experiment, where in vitro cell culture or ex vivo corneas were transfected with a GFP gene without vector-reagent, no GFP expression was detected (data not shown).

Tf-PEI Conjugate (DuoFect)

We tried several N/P ratios [molar ratio of nitrogen (PEI) and phosphate groups (DNA)] ranging from 3.6 to 6.0, with various amounts of DNA (from 5 μg to 50 μg) and modified the incubation time with the transfection complex from 4 hours up to 5 days (with no medium change). As a result, few CECs expressing GFP fluorescence were found, and the most were seen at a N/P ratio of 4.8, with 10 μg DNA after 72 hours of continuous incubation (Figure 1a). Some signs of cytotoxicity, such as impaired growth and elongated form of CECs (compared with control) were observed (Figure 1b). In ex vivo culture corneas, Tf-PEI-mediated transfection failed to show GFP expression in the endothelial cells (data not shown). Therefore, no further study using this vector was conducted.

FuGENE 6 and TransIT-LT

In Vitro Cell Culture

Initially, three reagents of TransIT-PanPak (TransIT-LT1, TransIT-LT2, and TransIT-Insecta) were tested. Of these, TransIT-LT1 proved to be the most effective (self-observation). Therefore, TransIT-LT1 was used in further experiments as a liposome of low toxicity. As the results of in vitro study demonstrated, FuGENE6 and TransIT-LT effectively mediated the transfer of the GFP gene into rabbit CECs (Figures 1c, d), showing both nucleic and cytoplasmic localization of GFP fluorescence. In the preliminary experiments, different combinations of DNA to vector-reagent ratios (w/v) ranging from 1/1 to 1/10 and various amounts of DNA (from 1 μg to 10 μg) were tried. We observed that FuGENE6 and TransIT-LT showed very similar behavior, and that the complex with DNA/vector ratio of 1/2 or 1/3 using 3 μg DNA per 1 mL of culture medium produced the best efficiency. To reduce potential toxicity we decided to use 1/2 DNA/vector ratio and 3 μg DNA per 1 mL of culture medium as the optimal concentration in further study. Comparing the efficiency obtained by transfection in serum-free and serum-added medium, we found that the presence of serum (FCS) yielded markedly higher productivity. Regarding cell density at time of transfection, we noticed that the cell cultures of 60–70% confluency yielded the best and most stable reproducibility. In addition, the incubation time with transfection complex was crucial. Few CECs expressed GFP fluorescence when the transfection complexes were removed and replaced with fresh medium at 4 hours, but the number of GFP-positive cells was dramatically increased when the medium was replaced after 24 hours. Even in continuous incubation, no apparent toxicity of the CECs was observed. In in vitro study, the number of CECs expressing GFP fluorescence was increasing from day 1 to day 3, about stable on days 3–5, and declining afterward (self-observation). The transfection efficiency of FuGENE6 and TransIT-LT estimated at day 3 was 28.8% and 38.8%, respectively (Figure 2, left).

Ex Vivo Culture

As the study in ex vivo culture showed, FuGENE6 and TransIT-LT also mediated the transfer of the GFP gene to the corneal endothelium, resulting in GFP fluorescence expression (Figures 1e, f). Ini-

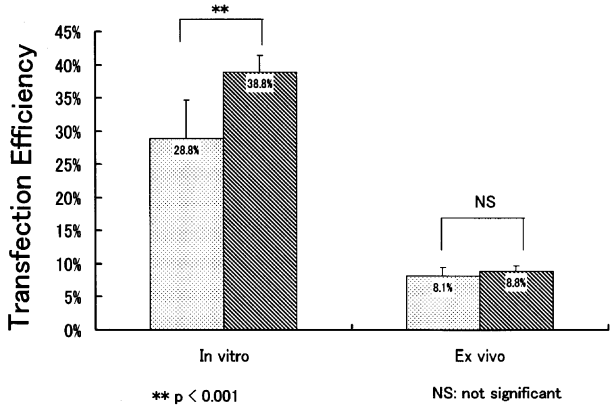

Figure 2. Transfection efficiency of FuGENE6 and TransIT-LT estimated at day 3 after transfection. The difference between FuGENE6 and TransIT-LT was statistically significant ($P < .001$) in vitro, but not significant in ex vivo culture. Data compiled from three independent experiments using three separate microscopic fields. □ FuGEN6; ▨ TransIT-LT.

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tially, other trials were also performed; however, the above optimal concentration and conditions in the in vitro study seemed most suitable for ex vivo culture. The kinetics of expression of the GFP reporter gene in ex vivo corneas was studied for up to 2 weeks. FuGENE6 and TransIT-LT showed very similar courses of GFP gene expression, where GFP fluorescence was detected in only a few CECs 24 hours after transfection, but maximally observed on days 3–5, and declined to undetectable level after 14 days (Figure 3). The efficiency of FuGENE6 and TransIT-LT in ex vivo culture estimated at day 3 was 8.1% and 8.8%, respectively (Figure 2, right). The expression of a GFP reporter gene was strictly limited to the corneal endothelium. When endothelium with Descemet's membrane was peeled off, no GFP fluorescence was detected either in the stroma or in the epithelium (data not shown).

Endothelium Viability

After 3 days of continuous incubation, in both FuGENE6- and TransIT-LT-treated corneal specimens, Trypan Blue staining did not reveal any blue (dead) cells in the endothelium (data not shown). No endothelial cell loss was observed.

Endothelium Morphology

The SEM images of the endothelium from FuGENE6- and TransIT-LT-treated corneas, which were incubated for 3 days without medium change, showed no apparent morphological alteration of CECs compared with the control (Figures 4a, b, c).

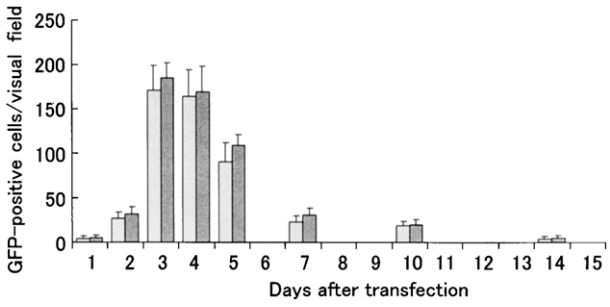

Figure 3. Time course of green fluorescent protein (GFP) expression. Ex vivo culture rabbit cornea was transfected with pEGFP-C1 coding for GFP fluorescence using FuGENE6 or TransIT-LT and continuously incubated in Dulbecco's modified eagle's medium with 10% fetal calf serum. The results represent the mean ± standard error of triplicate corneal specimens. (Here, in a microscopic field, there were approximately 2085 endothelial cells). □ FuGEN6; ▨ TransIT-LT.

Discussion

Tf-PEI conjugate (DuoFect) is a nonviral DNA delivery system based on transferrin receptor-mediated endocytosis to carry DNA into cells and utilizing the capacity of the cationic polymer PEI to mediate efficient gene transfer into a variety of cells.[13,14]

However, in the case of corneal endothelial cells, Tf-PEI conjugate showed limited efficacy in vitro and no effect ex vivo. The toxicity observed in vitro was probably due to the destabilization of the cell membrane by PEI.

FuGENE6, a new lipid formulation for the transfection of mammalian cells, relies on the interaction of a cationic lipid molecule with plasmid DNA to form a complex, which is taken up by the cells. After being released from the endosomes, the plasmid DNA enters the nucleus by a yet unknown mechanism.[15] TransIT-LT is a new generation of cationic-liposomes. The mechanism of the liposome transfection system represents endocytosis as a major route of intracellular gene delivery. Several features of cationic liposomes such as their ability to complex large amounts of DNA and versatility in use with any type and size of DNA or RNA have made them attractive for DNA delivery.[16] In this study, FuGENE6 and TransIT-LT could mediate GFP gene transfer effectively to the CECs both in in vitro and in ex vivo culture. In vitro, TransIT-LT was significantly more efficient than FuGENE6 (38.8% compared with 28.8%), while in ex vivo culture, their efficiency was almost the same. Although the efficiency in ex vivo was still low, certainly these DNA vectors can be successfully used for research in in vitro culture of CECs, taking into account such advantages as relatively high efficiency, availability and simple manipulation. In our study, pEGFP-C1 coding for GFP had been used as reporter gene and the signal from GFP does not have any enzymatic amplification; hence, the sensitivity of GFP may be lower than that for enzymatic reporters such as β-galactosidase, secreted placental alkaline phosphatase, and firefly luciferase. However, transfection efficiency could be increased by using these new DNA vectors in combination with transferrin, a widely distributed cell surface receptor present on most proliferating and nondividing cells[17,18] or by pharmacological manipulation of the corneas before transfection using chloroquine.[8] Employing the unusually potent translocation properties of the VP22 fusion protein following transfection is another possibility to improve the overall efficacy of these DNA vectors.[19,20]

Lipid and liposome formulations, in particular, recently developed generations such as FuGENE6 and

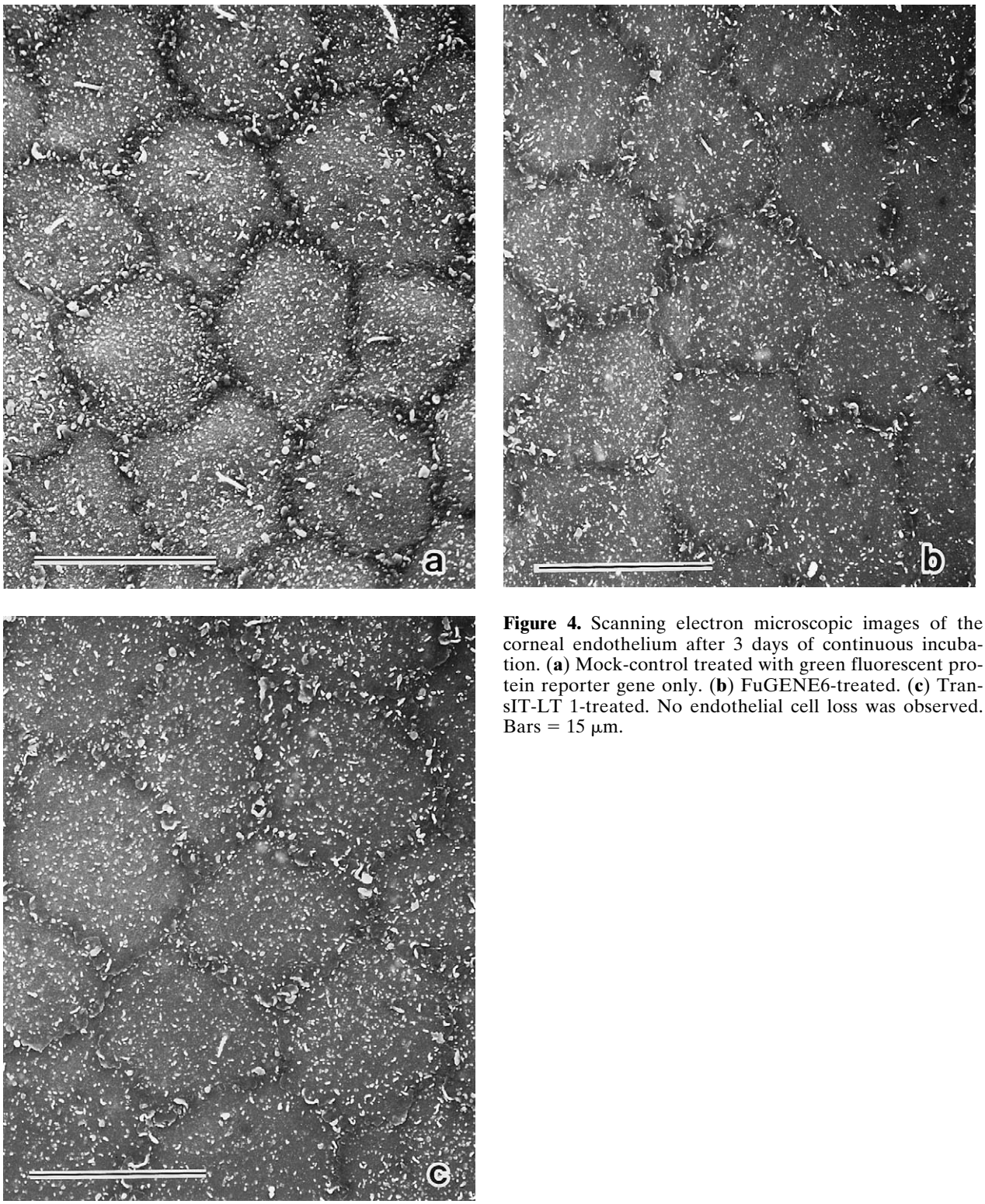

Figure 4. Scanning electron microscopic images of the corneal endothelium after 3 days of continuous incubation. (**a**) Mock-control treated with green fluorescent protein reporter gene only. (**b**) FuGENE6-treated. (**c**) TransIT-LT 1-treated. No endothelial cell loss was observed. Bars = 15 μm.

TransIT-LT are known for their low toxicity. The results of Trypan Blue staining and SEM showed good viability and no apparent morphological alteration of CECs. In addition, these systems are theoretically nonimmunogenic and are not accompanied by inflammatory responses, indicating the safety of their application.

Like in other delivery systems,[8,9] in FuGENE6- and TransIT-LT-mediated transfection, GFP reporter gene expression was detected in the endothelial cells only, indicating that transgene was selectively transferred to the corneal endothelium, but not to the epithelium (where vector access is not a problem) or stroma (which might have been a consequence of poor vector accessibility).

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We noticed that expression of the GFP reporter gene in ex vivo culture was short-term, lasting no more than 15 days in the rabbit; however, in some settings short-term gene expression might be desirable, avoiding potentially hazardous complications. The transient expression of transgenes may be safe and useful when gene therapy aims at inducing mitotic division or preventing cell loss during surgical procedures.

In conclusion, both FuGENE6 and TransIT-LT are safe, simple to use, and may provide useful alternative methods for gene transfer to the corneal endothelium, avoiding certain side effects of viral vectors. As their efficiency can be increased by various enhancement methods, these DNA vectors may be promising for practical application.

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