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Controllable Urokinase Gene Expression in Trabecular Meshwork Cells

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Abstract

Purpose: Glaucoma is a type of progressive optic neuropathy that finally leads to blindness related to elevated intraocular pressure (IOP). Accumulation of extracellular matrix in trabecular meshwork (TM) and juxta-canalicular connective tissues, which form an aqueous outflow pathway, may be a major cause of increased IOP, thus the fibrinolytic system may be associated with regulation of IOP. We examined the possibility of controllable urokinase plasminogen activator (uPA) gene transfer into TM cells.

Methods: TM cells were freshly isolated from porcine eyes and human TM cells obtained during trabeculectomy procedures and cultured. Total RNA was extracted from human TM cells and reverse transcribed into cDNA. A reverse-transcribed polymerase chain reaction (RT-PCR) method was then performed to detect the gene expression of uPA. The cDNA of human uPA was sub-cloned into an expression vector (pEYFP-N1 vector) and controllable expression vector (TRE-Tight vector), then each vector was independently transfected into cultured porcine TM cells. Doxycycline was added to the culture medium to activate the pTet-ON and TRE-Tight vectors. Finally, the expression of uPA was examined using an enzyme linked immunosorbent assay (ELISA).

Results: ELISA findings revealed the expression of uPA (3.5 ng/ml) in medium from cultured porcine TM cells that had been transfected with the human uPA gene using the pEYFP-N1 vector. Doxycycline induced human uPA in the pTet-On and TRE-Tight vectors with human uPA gene co-transfected TM cells in a dose-dependent manner.

Conclusions: Controllable gene transfer of uPA, which may degrade the extracellular matrix in juxta-canalicular connective tissue, into TM cells was achieved using a Tet-On system. The present method may be useful as a novel therapy for glaucoma.

Keywords: Glaucoma; Tet system; Urokinase; Trabecular meshwork cell

Introduction

Glaucoma is a type of progressive optic neuropathy that finally leads to blindness with disease advancement and affected patients experience gradually progressive visual field loss caused by irreversible optic nerve damage related to elevated intraocular pressure (IOP) [1]. IOP is determined by the balance of aqueous production and outflow facility, while an elevated level is usually caused by increased resistance in the outflow pathway [2]. Autopsy results of eye balls dissected from primary open angle glaucoma (POAG) patients have shown that loss of TM cells followed by substitution with extracellular matrix (ECM), which has been suggested to contribute to increased resistance to aqueous outflow, resulted in elevation of IOP in those cases [3,4]. Thus, accumulation of ECM in trabecular meshwork (TM) and juxta-canalicular connective tissues, which form an aqueous outflow pathway, may be a major cause of increased IOP.

On the other hand, fibrinolytic system proteins, such as tissue plasminogen activator (tPA) and urokinase (uPA), have been detected in TM tissues [5-7]. Furthermore, it was reported that the gene expressions of some fibrinolytic proteins in cultured TM tissues obtained from patients with POAG were different as compared to those in cultured normal TM tissues [8], and it was suggested that the fibrinolytic system may be associated with regulation of IOP. In fact, Perkinse et al. [9] reported that injection of plasmin into the anterior chambers of eyes in living cynomolgus monkeys caused a substantial and statistically significant increase in the coefficient of facility of aqueous outflow.

We speculated that activation of the fibrinolytic system in TM may dissolve ECM and lead to decreased aqueous outflow resistance. Although gene transfer may be a good method to achieve overexpression of fibrinolytic system protein, control of dosage and duration, as well as related complications are controversial. Recently, a controllable gene transfer technique known as the Tet-On system became available [10], which enables expression of gene products within an acceptable period of time and with proper dose response. Controllable fibrinolytic protein gene transfer in TM has not been previously reported. Here, we report that controllable uPA gene transfer into TM cells was achieved.

Materials and Methods

TM cell cultures

Porcine eyes were obtained from a local slaughterhouse within 60 minutes of death and transported to our laboratory. The eyeballs were cut at a point 5 mm posterior from the limbus, then the vitreous and lens were separated from the ciliary body and removed. The iris was incised and TM tissue was gently removed from the anterior chamber angle with a fine forceps under direct observation with a microscope. TM tissue explants were then incubated in Dulbecco's Modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12) (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS: HyCloneLaboratiries, South Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2/95% air at 37°C, and supplied with fresh medium every 3 days. In addition, after receiving informed consent according to the Helsinki Declaration, human TM tissue was removed from a POAG patient during a trabeculectomy procedure and cultured in the same medium as described above. After 2-week cultures, both human and porcine TM cells had migrated from their respective TM tissues and proliferated, and were collected using Hank's balanced salt solution containing 0.05% trypsin and 0.02% EDTA, then serially cultured. When the cells became confluent, they were sub-cultured at a split ratio of 1:3 and used after the 2nd or 3rd passage in the following experiments.

Identification of trabecular meshwork cells

Cells were examined for expression of the Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) receptor, which has been shown to be a surface marker for TM cells [11]. Cells were grown in 35 mm diameter glass-bottom dishes (Matsunami Glass Ind, Ltd. Japan) and incubated for 6 hours in 2 ml of medium containing 20 μg of Dil-labeled acetylated LDL (Dil-Ac-LDL; Molecular Probes, Eugene, OR). After washing the dishes 3 times with phosphate-buffered saline (PBS, pH 7.4), they were processed for fluorescence microscopy examinations and analyzed using a fluorescence microscope (FluoView, Olympus, Tokyo, Japan).

Reverse-transcribed polymerase chain reaction (RT-PCR) assays

The gene expression of uPA was examined using an RT-PCR method. Total RNA was extracted from human TM cells using TRizol® (Invitrogen Life Technologies, Carslbad, CA) and 1 μg of total RNA was reverse transcribed into cDNA using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). First-strand cDNA was amplified with Taq polymerase (Takara, Shiga, Japan) and anti-Taq high (TOYOBO, Osaka, Japan) using oligonucleotide primers specific for human uPA. The sequences of the oligonucleotide primers used in the PCR reactions were designed to obtain the full length of uPA gene cDNA (1296 bp), as follows: forward 5'-tcaccaccaaaatgctgtgt-3' and 5'-aggccattctcttccttggt-3'.

Subcloning of human uPA gene

The PCR product was purified and introduced into a pCR8/GW/TOPO vector (TA cloning kit, Invitrogen, Tokyo, Japan) to prepare the complete sequenced cDNA of human uPA.

Transfection

First, we used a pEYFP-N1 vector to easily detect whether the gene was transferred, as indicated by yellow fluorescence. The complete sequenced cDNA of human uPA was sub-cloned into a pEYFP-N1 vector and transfected into cultured porcine TM cells using Lipofectamine® 2000 Reagent (Invitrogen Life Technologies) as a transfection agent, following the manufacturer's instructions. As a control, a pEYFP-N1 vector alone was also transfected into cultured cells.

Next, we used a Tet-On system (Clontech Laboratories Inc., Mountain View, CA) to control uPA gene expression. Human uPA cDNA was introduced at the laboratories of Takara Dragon Genomics Corporation (Shiga, Japan), then TM cells transfected with the pTet-On vector were purified using G418 sulfate (Clontech) to select successfully transfected cells. Cells were grown until reaching sub-confluence, then a TRE-Tight vector with the human uPA gene (TRE-Tight uPA) was transfected using Lipofectamine® 2000 Reagent with the same method described above.

Induction of uPA using doxycycline

Porcine TM cells co-transfected with pTet-On and TRE-Tight uPA vectors were treated with 1, 5, or 10 μg/ml of doxycycline to induce gene expression of human uPA, which was determined by examining culture media.

Enzyme-linked immunosorbent assay (ELISA)

Porcine TM cells transfected with the human uPA (pEYFP-N1 uPA)were cultured in 6-well culture plates for 48 hours, then uPA concentrations in culture media were determined using an ELISA kit (ZYMUTEST uPA antigen; Hyphen BioMed, Andresy, France), according to the manufacturer's instructions. Porcine TM cells co-transfected with pTet-On and TRE-Tight uPA vectors were treated with 1, 5, or 10 μg /ml of doxycycline for 96 hours, then uPA concentrations in culture media were measured.

Results

TM cell identification

Figure 1 presents photographs of human (A) and porcine (B) TM tissue cultures. TM cells migrated from the TM tissues and proliferated after incubation in DMEM/F12 for 1 to 2 weeks. Figures 2A and 2C show confocal fluorescent microscopic images of Dil-Ac-LDL staining of cultured porcine and human TM cells, respectively, while Figures 2B and 2D show Nomarski microscopic views of the same cells depicted in Figures 2A and 2C, respectively. Most of the cells (>95%) were positive for Dil-Ac-LDL staining, indicating that they were TM cells with a high expression of the Ac-LDL receptor.

uPA gene expression in human TM cells

RT-PCR results revealed the gene expression of uPA in cultured TM cells obtained from a POAG patient (Figure 3, 1296 bp).

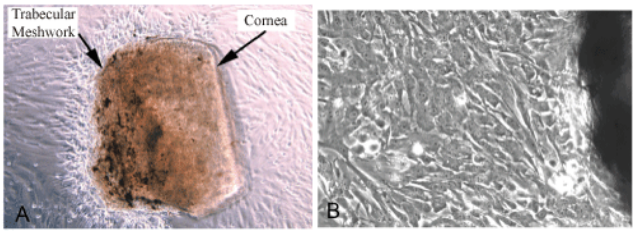

Figure 1: TM tissue cultures. A: Human TM tissues dissected from a glaucoma patient during trabeculectomy. Original magnification × 100. B: Porcine TM tissues. Both cultures exhibited migration and proliferation of surrounding TM tissues (arrows). Original magnification × 400.

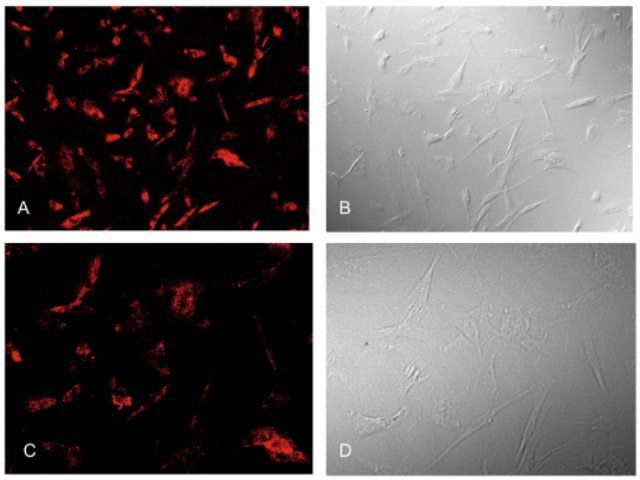

Figure 2: Dil-labeled LDL staining of cultured porcine (A, B) and human (C, D) TM cells. **A, C:** Cells were stained with Dil-labeled LDL and visualized with a confocal fluorescent microscope after 2-3 passages. **B, D:** Normarski view of the same cells. Original magnification × 400.

Gene transfer using pEYFP-N1 vector

Figure 4 indicates successful transfer of pEYFP-N1 vectors to cultured porcine TM cells. Fluorescence, as shown by enhanced yellow fluorescence protein (EYFP), was observed in TM cells transfected using Lipofectamine® 2000 (Figure 4).

Production of human uPA in porcine TM cells

ELISA findings showed that the uPA protein was detectable in culture media of porcine TM cells transfected with the human uPA gene using Lipofectamine® 2000, while it was not detectable in control cells transfected with only the pEYFP-N1 vector and without a human uPA construct (data not shown). The concentration of uPA in medium was calculated to be 3.5 ng/ml from a standard curve based on the gradient concentration of the human uPA protein (Table 1).

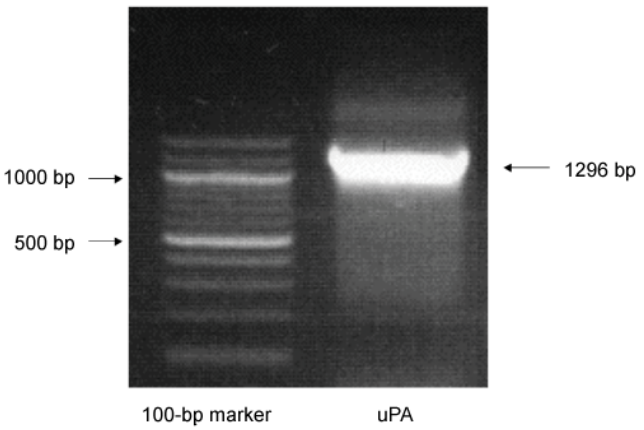

Figure 3: uPA gene expression in human TM cells. RT-PCR findings revealed the gene expression of uPA in cultured human TM cells.

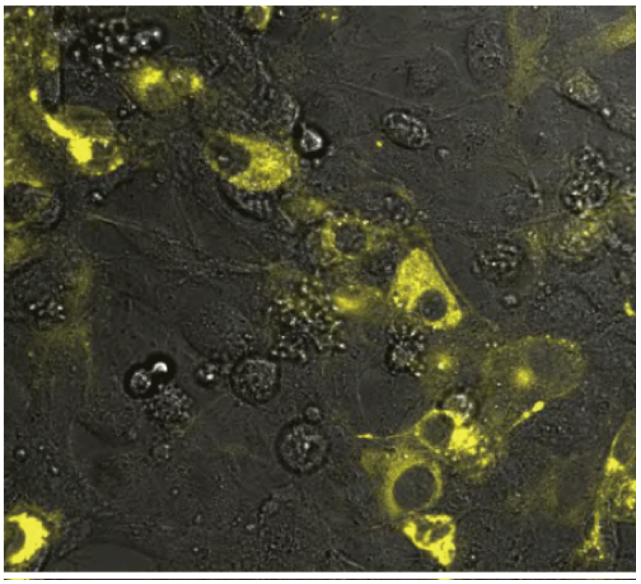

Figure 4: Gene transfer to cultured porcine TM cells using pEYFP-N1 vector. Yellow fluorescence was observed in EYFP-induced TM cells using Lipofectamine®.

Porcine TM cells were co-transfected with pTet-On and TRE-Tight uPA, then exposed to doxycycline at 1, 5, and 10 μg/ml, which resulted in0.07, 1.13, and 3.91 ng/ml, respectively, of human uPA in culture medium samples (Table 1). No detectable uPA was found in the control cultures.

Discussion

The present results demonstrate that uPA was successfully introduced into TM cells using a Tet-On system as well as a pEFYP-N1 vector. ELISA findings also revealed a controllable expression of transferred human uPA with bioactivity in the media of cultured

porcine TM cells that had been transfected with the human uPA gene using the Tet-On system.

TM cells are important for IOP lowering strategies, because the meshwork is a gateway to the aqueous outflow pathway. TM and juxta-canalicular connective tissues are both major causes of increased IOP, thus the TM is considered to be a good target for treatment of glaucoma in IOP lowering procedures. Historically, trabeculectomy surgical procedures have been used to reduce IOP by mechanical opening of TM tissues in glaucoma therapy [12-14], though it is impossible to eliminate the risk of loss of vision from complications such as infection [15] and bleeding [16]. In contrast, the present method may avoid various complications associated with such operations.

The Tet-On system, a sophisticated system for regulation of gene transfer, was established based on the finding of transcriptional activation by tetracycline and is currently available as a commercial product [10]. A pTet-On vector produces a specific product only with tetracycline or doxycycline in a dose-dependent manner. Furthermore, a TRE-Tight vector is activated by the pTet-On product and then shows dose-dependent expression of the co-introduced gene. In the present study, human uPA was successfully produced with the addition of doxycycline in a dose-dependent manner (Table 1).

Gene vector	pEYFP-N1 uPA	pTet-On/TRE-Tight uPA			
Doxycycline (μg/ml)	—	0	1	5	10
uPA (ng/ml)	3.5	0	0.17	1.13	3.91

Table 1: Summary of the results of ELISA for uPA.

ELISA: Enzyme-Linked Immunosorbent Assay; —: The dash represents no data collected; uPA: Urokinase Plasminogen Activator

It has been reported that the gene expressions of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases; TIMPs) were found in TM cells [17-23], and those expressions were shown to be regulated by mechanical stretching [19-21], liquid flow [22], and aqueous humor growth factor [23]. Additional studies are needed to examine these factors and determine if their addition would increase the efficiency of our technique.

In summary, we report successful controllable introduction of the uPA gene into TM cells *in vitro*. Further experiments using an *in vivo* glaucoma model will be important prior to clinical application.

Acknowledgments

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