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ORIGINAL PAPER

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In vitro and ex vivo gene delivery into proximal tubular cells by means of laser energy—a potential approach for curing cystinuria?

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Abstract Cystinuria is the cause of 1–2% of stones observed in adults and about 10% of those occurring in children. Recurrent stone formation and multiple operations cause considerable morbidity. We investigated the transfection efficiency of naked plasmid DNA in porcine kidney cells by applying holmium laser (Ho:YAG) energy in vitro as well as ex vivo in a porcine kidney papilla model. In the in vitro experiments, naked plasmid DNA was added to LLC-PK1 cells suspended in a medium and Ho:YAG laser applied with varying pulses. The transfection efficiency was measured by the expression of EGFP reporter gene in the cells by FACScan analysis and fluorescence microscopy. Ex vivo, papilla from porcine kidney was excised and naked plasmid DNA was added to the tissue in the medium. The laser was then applied and the cryosectioned tissue observed under fluorescence microscope. The efficiency of transfection in vitro significantly improved with the increase in impulses ($P < 0.01$). Transfection at 50 impulses averaged $0.7 \pm 0.3\%$, at 200 impulses $28.3 \pm 7.7\%$, and at 500 impulses $36.1 \pm 3.1\%$. The cell mortality rate increased with higher pulse rate up to 70%. Ex vivo trials showed transfection in extended regions of the tissue and also in the peripheral layers of the papilla. Our study indicates that the transfection of benign kidney cells by Ho:YAG is a promising new gene transfer strategy. The ex vivo trials showed that peripheral renal tissue layers are susceptible to transfection by Ho:YAG applied from the papillary surface.

Keywords Gene delivery · Laser energy · LLC-PK 1 · Transfection

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Introduction

Renal epithelium has a great impact on the regulation of urine composition. Several hereditary disorders involve the impairment of tubular secretion or reabsorption mechanisms such as cystinuria, which affects the luminal transport of cystine and dibasic amino acids in proximal tubules and the small intestine. It is characterized by excessive urinary excretion of cystine, arginine, lysine and ornithine. Cystine is the least soluble of all amino acids and its increased excretion results in frequent stone formation in the urinary tract leading to urinary obstruction, recurrent infection and the need for repeated intervention. This causes considerable morbidity and a high risk of developing renal insufficiency [1].

The results of medical and dietary treatment are unsatisfactory, therefore gene therapy might offer a causal treatment for such diseases. Two genes on chromosomes 2 (SLC3A1) and 19 (SLC7A9) have been identified, coding for parts of an amino acid transport system localized on the membranes of proximal tubule cells (PTCs) [2, 3]. The simplest idea of gene delivery is the use of naked DNA in the form of plasmids. However, efficiency is very low and limits its application. Lasers have been shown to improve transfection efficiency in vitro [4, 5, 6, 7]. In particular, the holmium:yttrium-aluminium-garnet laser (Ho:YAG), which is widely used in endourology, is a promising device that enhances gene delivery into target cells [8]. As kidneys are easily accessible by ureterorenoscopy, laser application might be a future possibility for selective, minimally invasive gene delivery. The aim of our study was to examine the possibility of delivering plasmids into proximal renal tubules in an in vitro and an ex vivo model.

Materials and methods

Cell culture

The LLC-PK1 (pig, *Sus scrofa*, normal kidney) cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). LLC-PK1 cells were grown in DMEM medium

(Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 2 mM glutamine (Invitrogen). They were routinely cultured to a confluence of 80%, seeded in T-75 flasks (BD Biosciences Clontech, Palo Alto, USA) and incubated at 37°C and 5% CO_2.

Reporter system used for transfection studies

Plasmid pEGFP-N1 (4.7 kb, BD Biosciences Clontech) encodes an enhanced variant of green fluorescent protein (GFP) of the *Aequorea victoria* green fluorescent gene that is human codon optimized for maximum expression in mammalian systems. The pEGFP-N1 plasmid DNA was purified using the QIAGEN-tip 10000 (Qiagen, Germany) according to the QIA Filter plasmid Giga protocol.

Transfection of cells by Ho:YAG laser

Cells were taken for the transfection studies at 80% confluence. They were trypsinized using trypsin-EDTA (0.25% trypsin, 1 mM EDTA, Invitrogen). The cell suspension was centrifuged for 5 min at 250 *g*. Cells were resuspended in FCS-free DMEM. They were then counted using a haemocytometer with trypan blue (Gibco BRL, Invitrogen). The final concentration of the cell suspension was adjusted to 3.5 million/ml. A measure of 1 ml of the cell suspension was then put into 5 ml NUNC CryoTube vials (Nalge Nunc International, Rochester, USA). A total of 50–500 µg of naked plasmid pEGFP-N1 DNA was added to each 1 ml of the cell suspension. A Ho:YAG laser (Wavelight Lasertech, Starnberg, Germany) was used. The laser energy was imparted to cells by applying energy pulses into the Falcon tube. Studies on transfection efficiency were carried out considering various parameters such as the energy level (2,000 mJ), the number of impulses (50–750), and DNA concentration (50–500 µg). Impulse frequency was adjusted to 10 Hz in accordance with preliminary experiments [8].

Analysis of transfection efficiency

The mortality rate of the cells after the application of laser energy was determined by haemocytometer and trypan blue. The laser-treated cells were cultured in a T-25 (BD Falcon) with 5 ml of the complete DMEM medium (10% FCS, 2 mM glutamine) and incubated at 37°C and 5% CO_2. The cells were analyzed by FACS for the percentage of transfection after 24 and 48 h of incubation. After incubation, the cells were observed under a fluorescence microscope and EGFP expression visualized in the transfected cells. The number of transfected cells was determined using a FACScalibur (Becton-Dickinson, Palo Alto, USA). Briefly, the cells were trypsinized and washed twice with phosphate-buffered saline (PBS, Invitrogen). They were then resuspended in 500 µl of PBS in a FACS tube (BD Falcon) for FACS measurement. The parameters for the flow cytometric analysis were set using cell suspensions as a negative control, which were not subjected to the laser treatment. For the transfection experiments, negative controls (cell suspension with naked DNA only) were used to determine the efficiency of the laser in relation to naked DNA delivery. Raw data obtained from flow cytometer by the Cell Quest program were interpreted by the WinMDI 2.8 software (TSRI, San Diego, USA). Lipofectamine 2000 based transfection was used as a positive control to determine the transfection efficiency in LLC-PK1 cells (Invitrogen). A standard Lipofectamine (manufacturer's) protocol was used for this purpose.

Ex vivo porcine papilla model

Fresh porcine kidneys were obtained and the papillae excised. The papillae sections were placed in 5 ml NUNC CryoTube vials containing normal DMEM medium. A volume of 300 µg of naked plasmid pEGFP-N1 DNA was added to the vials. Holmium laser energy was imparted to the papilla by applying energy pulses into the modified Falcon tube. The laser fiber was fixed by a micromanipulator either in a contact or non-contact mode with 2 mm distance between the tip of the fiber and the papillary surface (Fig. 1). After laser application, the tissues were placed in Petri dishes containing fresh, normal DMEM and incubated at 37°C and 5% CO_2. The papillae were sectioned using a cryostat (Cryostat HM500, Microm, Walldorf, Germany) and studied for the efficiency of transfection by fluorescence microscopy and phase contrast microscopy under a fluorescence microscope (Axioscop 2 plus, Zeiss, Germany).

Data calculation and statistical evaluation

Data are shown as mean ± standard deviation. Differences between groups were assessed by the Kruskal-Wallis test. SAS statistical software was used (SAS, Heidelberg).

Results

In vitro transfection

The transfection rates increased significantly between 50 and 500 impulses as shown in Fig. 2 ($P < 0.01$). Additionally, the duration of reincubation after transfec-

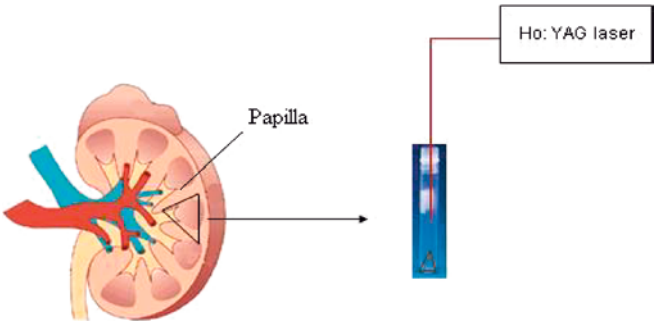

Fig. 1 Schematic figure illustrating the application of Ho:YAG laser to the porcine papilla ex vivo

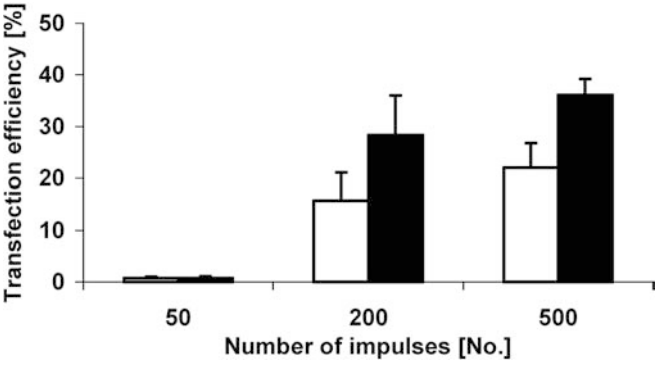

Fig. 2 Effect of number of impulses of holmium laser on transfection of LLC-PK1 cells. FACS results after 50, 200 and 500 impulses with 24 or 48 h of incubation are shown (24 h *white bars*, 48 h *black bars*; percentage of transfected cells, *bars* indicate SD)

tion had a significant effect on transfection efficiency. Although the transfection rate was $0.7 \pm 0.3\%$ (no significant difference compared to the negative control) after both 24 and 48 h of transfection with 50 impulses, in all other transfection experiments, a significantly higher transfection rates were obtained after 48 h reincubation (Fig. 2). All further experiments were therefore performed with 48 h reincubation. With 200 impulses, a great improvement in transfection rate to $28.3 \pm 7.7\%$ was observable. Application of 500 impulses led to a further moderate improvement ($36.1 \pm 3.1\%$). No additional increase in transfection efficiency was seen with 750 impulses. However, as a consequence of high impulse rates, the cell survival rate also decreased significantly from 80% at 50 impulses to 45% at 200 impulses and 30% at 500 impulses ($P < 0.05$).

The concentration of the plasmid DNA also showed a positive effect on the transfection efficiency. We reached our best results with a DNA concentrations of 300 µg/ml, which showed higher transfection rates in comparison to 50, 100 or 200 µg/ ml of plasmid DNA concentration. Very high plasmid amounts of up to 500 µg did not further optimize transfection efficiency (undisclosed results). In summary, a setting with 10 Hz repetition rate, 2,000 mJ laser energy and 200 impulses was the optimum for a good transfection efficiency in LLC-PK1 cells. For these parameter settings, the results were highly consistent with repeated trials. A representative FACS analysis with these parameters and variable impulses from 50–200 is shown in Fig. 3. These results were confirmed by fluorescence microscopy of the laser-treated cells that showed a comparable percent of fluorescent cells indicating successful transfection (Fig. 4). The negative controls showed transfection efficiencies of less than 1%. Lipofectamine, used as a positive control, revealed transfection rates of $62.7 \pm 2.7\%$ after 24 h of incubation.

Ex vivo transfection

Preliminary experiments showed a high mortality rate when laser energy was applied with tissue contact. Therefore, all further experiments were performed with the laser fiber being fixed by a micromanipulator to maintain a 2 mm safety distance between the tip of the laser fiber and the papillary surface. With this distance, coagulation effects were usually not observed. Fluorescence microscopy of the cryostat papilla sections revealed successful transfection. The analysis revealed very high GFP expression in the inner layers of the papillae. Furthermore, in most sections, plasmid penetration was also seen to some extent even in the outer layers of the kidney tissue (Fig. 5). Although we were not able to quantify cell transfection using our technique, it was evident that laser energy was capable of promoting DNA transfer from the collecting system through most of the renal tissue.

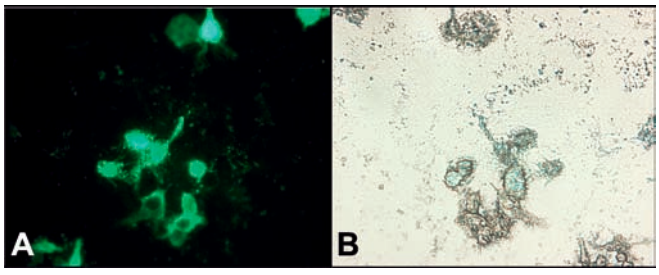

Fig. 4 Fluorescence microscopic images: **A** LLC-PK1 cells transfected by holmium laser in comparison with **B** phase contrast image (20×)

Discussion

The technically most simple form of gene therapy is the use of naked DNA. It is generally administered as a plasmid containing the gene associated with an appropriate promoter. The major limitation in translating this concept of gene therapy to clinical use is the low efficiency of gene transduction and undirected delivery to the target cells. Therefore, getting the gene to the target cell with a high transfection rate is a subject of great interest. Relatively new delivery approaches like elec-

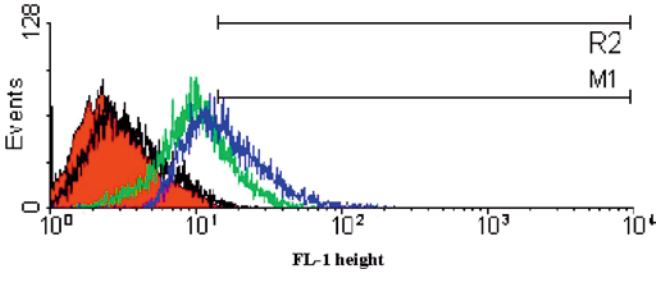

Fig. 3 FACS results for Ho:YAG transfection at optimal energy settings (10 Hz repetition rate, 2,000 mJ energy, 300 µg DNA, 24 h incubation) and 50 (*black*), 200 (*green*) and 500 (*blue*) impulses, negative control (*red*)

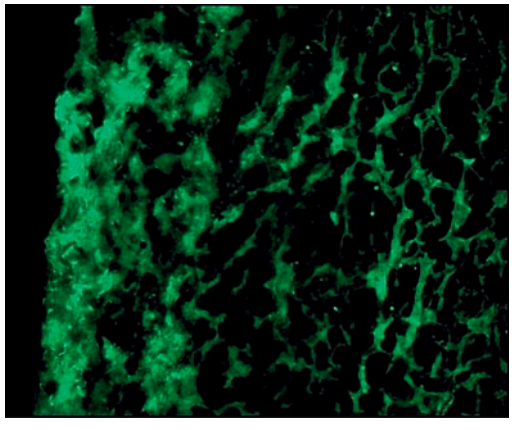

Fig. 5 Transfection of porcine papilla by Ho/YAG (10 Hz repetition rate, 2,000 mJ energy, 300 µg DNA, 24 h incubation, 10×)

troporation, hydrodynamics and ultrasound have been shown to enhance the expression of naked plasmid DNA in a variety of tissues [4, 5 ,6, 7, 8, 9, 10]. Among the described methods, electroporation has been used extensively, though it is highly disruptive and causes large scale cellular death [7]. Lasers have been shown to be efficient for the introduction of foreign DNA into cultured cells [9]. A cultured cell perforated with a finely focused laser beam was found to repair itself within a short period of time. Palumbo et al. showed that gene transfer in eukaryotic cells was possible by dye-assisted laser optoporation [4]. They also noted that the permeability of the cell membrane is modified at the site of beam impact. Shirahata et al. observed a similar effect of gene transfer when a pulse laser irradiation was focused on a cell membrane [5]. These studies have shown that upon laser irradiation the cells undergo a change in permeability of the plasma membrane or form pores in the membrane at the site of contact. These changes are transient and do not appear to damage the cells extensively. However, only low rates of gene delivery have been achieved. In our laboratory, we were able to confirm the efficacy of laser enhanced gene transfection not only in single cells but also in a cell suspension of tumor cell lines [8, 10]. These experiments showed excellent transfection rates exceeding 40%, which are, to our knowledge, the highest reported so far for experiments with naked plasmid DNA [10]. We demonstrated, that the Ho:YAG laser, which is routinely used in endourology, was more effective in cell transfection than the Neodymium:YAG laser. Based on our trials, transfection efficiency in LLC-PK1 cells was dependent on several parameters: laser energy, number of impulses, DNA concentration and time of incubation after laser transfection. For the current experiments with LLC-PK1 cells, the transfection rates achieved were less compared to the tumor cells, but still very efficient. An explanation might be that well differentiated benign cells are more resistant to DNA transfer methods than malignant cells with a changed physiological pattern. To our knowledge such differences have not been reported previously.

Transfection rates of up to 36% were observed for LLC-PK1 in vitro with higher impulses. However, the mortality rates of the cells also rose with an increase in impulses. This could be because the energy at such high impulse rates can affect the LLC-PK1 cells unpredictably. As reported by Palumbo et al. and Shirahata et al., excessive laser energy might alter the cell membrane properties and lead to cell death [4, 5]. In earlier experiments, we demonstrated that a laser energy of 2,000 mJ is necessary for most efficient gene transfer. Although cell mortality was significantly decreased, lower energy levels did not reveal satisfactory transfection results. In contrast to other laser transfection experiments, which were carried out on single cells [6, 7], we performed our transfection studies using cell suspensions. It is conceivable that cells are injured by induced turbulence within the tube during laser application. The observation of an improved transfection rate after 48 compared to 24 h may be explained by cell repair mechanisms in the intervening time interval. Although the transfection efficiency and cell death rate have to be optimized, our results are, to our knowledge, the first demonstrating successful laser-enhanced delivery of naked DNA in benign renal cells.

In our ex vivo experiments with porcine papilla tissue, we observed good transfection in the papillary layers (Fig. 5). In addition, moderate transfection was also seen throughout the tissue, even in the peripheral layers. Though there was a marked loss of tissue when the laser was applied in a contact mode, laser application in non-contact mode only moderately affected the tissue. These results verify the possibility of using the Ho:YAG laser as a new method of gene transfection not only in cells but also in intact tissues.

Our excellent gene transfection rates with the Ho:YAG laser in LLC-PK1 cells and porcine papilla underline the potential of this method for gene therapy to the kidney with minimal invasiveness. We have shown that the holmium laser is able to transfect not only tumor cells but also benign cells with high efficiencies. Our results are promising and the possibility of correcting dysfunction of the tubular amino acid transport in the cystinuria phenotype, with minimal invasiveness, warrants further in vivo studies.

References

1. Gambaro G, Favaro S, D'Angelo A (2001) The risk of renal failure in nephrolithiasis. Am J Kidney Dis 37:233–243
2. Calonge MJ, Gasparini P, Chillaron J, Chillon M, Gallucci M, Rousaud F, Zelante L, Testar X, Dallapiccola B, Di Silverio F (1994) Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. Nat Genet 6:420–425
3. Fernández E, Carrascal M, Rousaud F et al. (2002) rBAT-b0,$^{+}$ AT heterodimer is the main apical reabsorption system for cystine in the kidney. Am J Physiol Renal Physiol 283:F540-F548
4. Palumbo G, Caruso M, Crescenzi E, Tecce MF et al. (1996) Targeted gene transfer in eukaryotic cells by dye-assisted laser optoporation. J Photochem Photobiol 36:41
5. Shirahata Y, Ohkohchi N, Itayak H, Satomi S (2001) New technique for gene transfection using laser irradiation. J Inv Med 49:184
6. Schneckenburger H, Hendinger A, Sailer R, Strauss WS et al. (2002) Laser assisted optoporation of single cells. J Biomed Opt 7:410
7. Tirlapur UK, Konig K (2002) Targeted transfection by femtosecond laser. Nature 418:290.
8. Knoll T, Trojan L, Langbein S, Sagi S, Alken P, Michel MS (2004) Impact of holmium:YAG and neodymium:YAG lasers on efficacy of DNA delivery in transitional cell carcinoma. Lasers Med Sci (in press)
9. Kurata S, Tsukakoshi M, Kasuya T, Ikawa Y 1986) The laser method for efficient introduction of foreign DNA into cultured cells. Exp Cell Res 162:372
10. Sagi S, Knoll T, Trojan L, Schaaf A, Alken P, Michel MS (2003) Gene delivery into prostate cancer cells by holmium laser application. Prostate Cancer Prostatic Dis 6:127–130