Acoustic Energy: A New Transfection Method for Cancer of the Prostate, Cancer of the Bladder and Benign Kidney Cells

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Abstract. Background: Clinical use of gene therapy is limited by the poor efficacy and accuracy of intracellular DNA delivery. Known concepts of DNA transfection have not yet become clinical routine in the treatment of human disorders. We therefore focused on new transfection methods using different forms of acoustic energy as potentially safe and topographically applicable methods for gene delivery in the field of urology.

Materials and Methods: Three different cell lines (prostatic and urothelial cancer, benign kidney) were transfected by different forms of acoustic energy. The effect of several parameters of electromagnetic shock wave treatment (number and frequency of impulses, energy flow density and plasmid concentration) as well as focused ultrasound on the transfection rate was assessed in a standardized experimental setup. The transfection rate was measured through reporter genes (pEGFP) by FACScan. Transfection by lipofectamine and electroporation served as positive controls. Results: All cell lines were transfectable by acoustic energy. Maximum transfection rate was achieved using focused ultrasound (49.5%; 200 W, 500 ms, 200 μg/ml DNA). 31.3% of kidney cells were transfected by electromagnetic shock waves (1500 impulses, 200 μg/ml DNA, 0.5 mJ/mm² energy density, 2 Hz). Plasmid strand breaks were identified as a limiting factor of the transfection rate. Conclusion: Transfection by acoustic energy, especially focused ultrasound, can be achieved at a high level in different cell lines. The possible topical application to urological organs and the low level of side-effects make acoustic energy a promising new gene therapy treatment option in urology.

A prerequisite for gene therapy is the transfer of DNA into the cell (1). Various DNA transfection strategies exist, including viral reporter genes (2), nuclear microinjection (3), electro-transfection (4) and gene gun-mediated transfection (5). Transfection by retroviral vectors can cause problems, there may be limitations of the length of DNA sequences and possible negative side-effects from immunological reactions (1). Known concepts of chemical and physical transfection have not demonstrated a breakthrough in gene delivery in vivo to date. The specificity and selectivity of these techniques were not satisfactory (1). All concepts were inhibited by the inefficiency and inaccuracy of gene delivery into target cells and the negative side-effects of the method itself. At present, most of the common transfection methods are therefore unsuitable for in vivo application in humans (6). Nevertheless, it is still expected that gene therapy will play a major role in coming years in the treatment of hereditary disorders such as cystinuria or acquired disorders such as prostate or bladder cancer.

It has been demonstrated that DNA transfection is effectively enhanced by the use of physical methods such as ultrasound and shock waves. Several studies reported increased gene transfection into cells by the permeabilization of cell membrane with high-energy acoustic waves (7,8). Anatomically, it is possible to apply acoustic energy to major urological organs, e.g. prostate, bladder and kidney. Unfortunately, there is no precise in vivo information on the different forms of acoustic energy, converging parameters and consecutive effects on transfection and cell survival rate.

We attempted to evaluate the efficacy of two different forms of acoustic energy (electromagnetic shock waves and focused ultrasound) at various settings in DNA transfection. The aim was to establish a standardized protocol for three "urological" cell lines, with optimal transfection and cell survival rates, to serve as a template for further in vivo studies in animal models.

Materials and Methods

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The cells were cultured in a 5% CO₂ incubator, 100% air humidity atmosphere at 37°C. MatLu cells were maintained in RPMI 1640 media (Gibco BRL, Life Technologies, Rockville, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 4 mM l-glutamine (Gibco BRL) and 250 nM dexamethasone (Sigma, St. Louis, USA); RT-112 and COS-7: DMEM media (Gibco BRL), 10% FBS and 4 mM l-glutamine. Subcultivation was performed at 60% confluence by trypsinization and resuspension.

The cells were subcultured up to 30% confluence 24 h prior to the transfection experiments. After trypsinization and centrifugation (250 x g, 5 min), cell density was adjusted to 3.5x10⁶ cells/ml with phosphate-buffered saline (PBS, Gibco BRL).

All experiments were conducted 8 times for each different cell preparation.

**Reporter plasmids.** Enhanced green fluorescent protein plasmid (pEGFP, Clonetech, Palo Alto, USA) was used as a reporter gene to detect transfected cells. The plasmid was cloned into *E. coli* DH5α (high copy number plasmid) as a Glycin-Stock (kindly provided by Prof. Hafner, University of Applied Sciences, Mannheim, Germany). For preparation, supercoiled DNA was purified from *E. coli* by standard method (Qiafilter-Plasmid-Gigakit, Qiagen, CA, USA). The purified DNA was dissolved in TAE buffer (Gibco BRL) and stored at -20°C until use.

**Transfection by acoustic energy.** All cell-plasmid suspensions of 1 ml were transferred into 2.7 ml propylene Cryo Tubes (Nalge Nunc International, Roskilde, Denmark). Two different generators were used for in vitro transfection by acoustic energy.

The experimental shock wave generator Modulith SLK (Storz Medical, Kreuzlingen, Switzerland) was the source of the electromagnetic shock waves. The shock wave probe was placed under a plastic reservoir. The floor of the reservoir was covered with a plastic film to enable transmission of the shock waves into the reservoir. The probe was coupled by the use of ultrasound gel. The tubes were fully submerged into degassed water. The samples were focused under ultrasound monitoring (3.5 MHz). The generator settings were varied: number of impulses (500, 1000, 1500 and 2000), energy density (0.1, 0.25 and 0.5 mJ/mm²), frequency (1, 2 and 4 Hz), and three different plasmid concentrations were used (100, 150 and 200 µg/ml).

Secondly, focused ultrasound was delivered to the cell-plasmid suspension. The generator (Storz Medical) was specially designed for experimental investigations. Parabolic reflectors produce a focus at 100 mm distance to the piezo-electric element with a frequency of 1.07 MHz. The probe was set in a 70 liter tank with focus 5 cm under the water surface. As described above, samples were placed in the focus. The effect of three different power levels on MatLu cells was evaluated (100, 200 and 300 W; constant pulse time: 500 ms; inter-pulse period: 10 sec). Overall 10 impulses were applied for each sample.

Agarose gel analysis was performed to assess the preparation and quality of the plasmid as well as plasmid integrity after shock wave treatment. One percent agarose gel (Gibco BRL) and 50 ml tris acetate EDTA (TAE) buffer were microwave-heated, 0.5 µl ethidium bromide (Sigma) added and then filled into a gel chamber (B1A, PeqLab, Erlangen, Germany) at 50°C. Samples in 10 µl loading buffer were electrophoresed separated for 15 min at 100 Volts (TWPS Ilkrich, Appligene, France). Bands were photographically documented under UV light (TFL 20M, Appligene).

**Positive control group.** All three cell lines were transfected by the use of Lipofectamine (Life Technologies) in 6-well plates (Falcon, BD Biosciences, Palo Alto, USA). Prior transfection 250,000 cells each were cultured for 24 h in 2 ml of media and were washed with SFM (serum-free media). Two µg/ml of DNA and 10 µg/ml of Lipofectamine were prepared in 100 µl SFM (Cos-7: 375 µl) and then incubated for 30 min. Solutions were suspended with 800 µl SFM and incubated for 5 h on prepared cells. Media was changed to 1 ml complete media incl. 20% FCS.

Second, electroporation was used as positive control (Gene-Pulser II, Bio-Rad, Hercules, CA, USA). Cells were treated when
70-80% confluent. Washed in HeBS buffer (Hepes buffered saline, Sigma), cells were trypsinized. After centrifugation (5 min, 250 x g) cells were resuspended in HeBS buffer and the density was adjusted to 2x10^4 cells/ml HeBS buffer. Plasmid DNA was added at a concentration of 50 µg/ml. Electroporation (250 V, 950 µF) was performed in 4-mm electroporation cuvettes (EquiBio, Boughton Manchelsea, Kent, UK) containing 500 µl cell suspension each. The cells were allowed to incubate for 10 min after electroporation. The cells were resuspended in appropriate media. After incubation for 16 h, they were washed with PBS and incubated for a further 8 h. All samples were analyzed by FACScan for expression of EGFP.

**Results**

The aim of the study was to evaluate the rate of DNA transfection in cell lines by acoustic energy from two generators with different parameter settings. All three cell lines were transfectable by the two forms of acoustic energy. 

**Electromagnetic shock waves.** The impulse rate had a significant influence on the transfection rate of all three cell lines. Other parameters were kept constant: 200 µg/ml plasmid DNA, energy density: 0.5 mJ/mm², frequency: 2 Hz. With the exception of one sample, transfection was detected in all analyzed cell lines with 4 different impulse settings. In the exception, the survival rate of COS-7 was 0% after shock wave treatment at 2000 impulses and therefore it was impossible to assess transfection.

The rising number of impulses resulted in a distinct linear increase of the transfection rate in all cell lines (MatLu: p<0.0001, COS-7: p=0.0015, RT-112: p=0.004). In contrast, the cell survival rate declined in all three cell lines. The maximum rate of transfection was achieved in COS-7 cells at 1500 impulses (31.3%), whereas at 2000 impulses the transfection rate in MatLu cells was 24.9% and in RT-112 13.37%. The achieved transfection rates and cell survival are summarized in Figure 2.

**Statistical analysis.** Differences in the transfection or cell survival rate between groups with diverse parameter settings were determined using the Kruskal-Wallis (rank sum) test. One-sided p<0.05 was considered to be significant. Statistical analyses including calculation of mean value (MV) and standard deviation (SD) were performed by SAS System (Release 8.02; SAS Institute Inc., Cary, NC, USA).
Changes in generator energy density – other mentioned parameters were kept constant – significantly affected the transfection and survival rates of MatLu cells. Energy density settings ranged between 0.1 and 0.5 mJ/mm² (plasmid concentration: 200 µg/ml, impulse rate: 1000). The transfection rate increased significantly by 9.1% (11.2 to 20.3%, \( p = 0.0002 \)), whereas the cell survival rate declined dramatically by 62.9% (94.5 to 31.6%, \( p = 0.003 \)) (Figure 3).

Frequency only slightly affected the transfection rate of MatLu cells: the transfection increased from 16.3% (1 Hz) to 19.6% (4 Hz). Frequency change only negligibly affected the cell survival rate with 2% difference between 1 and 4 Hz.

The cell survival rate in MatLu cells remained constant at plasmid concentrations ranging between 100 and 200 µg/ml. On the other hand, different plasmid concentrations significantly affected the DNA transfection rate \( (p = 0.0005) \) in MatLu cells, resulting in an increase of 7.6% with a maximum transfection rate of 20.2% (200 µg/ml DNA). The data is summarized in Figure 4.

The rising number of impulses of electromagnetic shock waves (frequency: 2 Hz; 0.5 mJ/mm² energy density; 200 µg/ml DNA concentration) causes part-fragmentation of pEGFP plasmids. An example of plasmid fragmentation assessed by agarose gel analysis is shown in Figure 5.

**Focused ultrasound.** In contrast to previously analyzed parameters, the generator power of focused ultrasound resulted in a non-linear rate of transfection. Maximum transfection was achieved at 200 W (49.5%) with lower rates at 100 and 300 W (29.0% and 6.4%, respectively). The
elevation of power caused a drop from 56% to 17% in the cell survival rate. Transfection rate and cell survival rate are depicted in Figure 6.

Control groups. Transfection by lipofectamine was time-dependent in all three cell lines. For this reason, incubation periods from 18 up to 72 h were analyzed. The longer the incubation period, the greater was the increase in transfection rate observed in all three cell lines (data not shown). Samples contained 250x10^3 cells, a DNA concentration of 2.0 µg/ml and 10 µg/ml lipofectamine. Maximum transfection was achieved with COS-7 cells (35.7%), followed by MatLu (32.9%) and RT-112 cells (21.0%) after a 72-h incubation.

Using electroporation, a maximum transfection rate of 27.4% was achieved in COS-7 cells, MatLu cells displayed a medium transfection rate of 21.8% and RT-112 a rate of 14.8%.

Data from the two control groups is summarized in Figure 7.

Negative controls (incubation with pEGFP for 16 hours only) displayed a transfection rate of 0.1 to 0.3%.

Discussion

Benchmarks in the evaluation of different methods of transfection are the efficacy, in terms of transfection rate, and accuracy of gene delivery and possible negative side-effects. The clinical introduction of common procedures is problematic with respect to efficient gene delivery and tolerable side-effects. A key problem are the negative systemic side-effects that arise from most methods that do not allow the topical application of DNA transfection.

Therefore acoustic energy (shock wave and focused ultrasound) is of special interest in gene transfection therapy. Shock waves as well as focused ultrasound can penetrate deeply into the human body, thereby making the main urological organs such as bladder, prostate or kidney accessible for treatment (11). Extracorporeal shock wave lithotripsy is a widely accepted treatment option for humans and urologists already command the greatest skill and experience with this technique. Adverse clinical effects of this standard technique are rare (12). However, very few studies with transfection by acoustic energy exist to date. The aim of this study was, therefore, to evaluate the possibilities of transfection by high-density acoustic energy. Our special interest was to establish a standardized experimental basis for transfection in order to allow repeatable and – in contrast to previous reports (13) – direct cellular quantification of transfection by FACScan.

Our results confirm that both electromagnetic and focused ultrasound significantly enhance plasmid transfection in vitro. We demonstrated that, with optimal experimental parameter settings, it is possible to achieve transfection rates of up to 49.5% (MatLu) and 31.3% (COS-7) with focused ultrasound and electromagnetic shock waves, respectively. Transfection rates of HeLa cells in the range of 0.08% (shock wave) to 3% (focused ultrasound) have been recounted in published literature (13). The difference in cells, generators and experimental settings could account for the reasonable difference in transfection rates. The results of our investigations identify focused ultrasound to be a powerful new tool and possible new approach to gene transfection in...
bladder, prostate and renal tissue. The strand breaks, detected by gel electrophoresis, inhibit DNA transfection by acoustic energy. These strand breaks were caused by the rising number of impulses in treatment with electromagnetic shock waves. One subject to debate could be the possibility of combining acoustic energy for transfection with DNA repair inhibitors.

Common and efficient methods of transfection are those using lipofectamine and electroporation. To gain an insight into the relevance of our transfection rates achieved with acoustic energy and also for intercomparison, all three cell lines were also transfected by these two latter methods. In general, transfection by lipofectamine was more efficient than transfection by electroporation. With the investigated cell lines, transfection by focused ultrasound can enhance DNA uptake even more efficiently than lipofectamine (49.5% vs. 35.7% maximum transfection rate). Electromagnetic shock wave treatment shows comparable transfection rates to lipofectamine in COS-7 cells (31.3% vs. 35.7%), whereas RT-112 are transfected most efficiently by lipofectamine (13.4% vs. 22.0%). Unfortunately, lipofectamine and electroporation are not widely used for the intercomparison of transfection studies. In our opinion, this is a useful method of comparing experimental results.

Conclusion

Our results confirmed that the transient transfection of reporter genes into different cells from urological organs can be mediated by new forms of high-density acoustic energy. High transfection rates are achieved by the application of focused ultrasound in particular. Our results encourage further investigations into the application of acoustic energy and gene therapy in urology.

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