Study of Gene Transfection Using Laser Irradiation and Increased Temperature Techniques

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Abstract: Gene therapy can be defined as the deliberate transfer of DNA for therapeutic purposes. The scientific objective of this study is to improve the gene transfer by laser method: (1) To develop the gene transfection system using laser beams. (2) To test the energy-transformation relationship between laser energy and gene transfer. (3) To make test the time-transformation relationship between laser time accumulation and gene transfer. (4) To get influence of laser on the cells after gene transfer. (5) To transfer genes into cells with laser method. (6) To transfer genes into worms in vivo. (7) To transfer genes into animal bodies in vivo. (8) Clinical trial to the gene therapy. [Nature and Science. 2007;5(3):87-90]. (ISSN: 1545-0740).

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1. Introduction

Gene therapy has reached a crossroads during the past years (Matsui, 2003). Gene therapy can be defined as the deliberate transfer of DNA for therapeutic purposes. There is a further implication in that it involves only specific sequences containing relevant genetic information. Transplantation procedures involving bone marrow, kidney and liver are not considered a form of gene therapy. The concept of transfer of genetic information as a practical clinical tool arose from the gene cloning technology developed during the 1970s (Bechtel, 1979). Without the ability to isolate and replicate defined genetic sequences it would be impossible to produce purified material for clinical use. The drive for the practical application of this technology came from the biotechnology industry, with its quest for complex human biomolecules produced by recombinant techniques in bacterial. Within a decade, pharmaceutical-grade insulin, interferon, interleukin-2 (IL-2) and tumor necrosis factor (TNF) were all undergoing clinical trials. The next step was to obtain gene expression *in vivo*. Genetic disorders were the obvious first target for such therapies. Abortive attempts were made in the early 1980s to treat two patients with thalassaemia (Temple, 1982). These experiments were surrounded by controversy as the pre-clinical evidence of effectiveness was not adequate and full ethical approval had not been given. For the features of a suitable target disease for gene therapy approaches, certain factors should be considered. The disease must be life-threatening so that the potential risk of serous side-effects is ethically acceptable. The gene must be must be available and its delivery to the relevant tissue feasible. This may involve the ex vivo transfection or transduction of cells removed from a patient, which are returned after maniputation. This approach is only possible with a limited range of tissues and most trials so far have used bone marrow. Ideally, a short-tern surrogate end-point to demonstrate the physiological benefit of the newly inserted gene should be available. The electrical conductance change in the nasal epithelium after insertion of the cystic fibrosis trans-membrane regulator gene is a good example. Finally, there must be some possibility that the disability caused by a disease is reversible. Some of the tragic mental and physical handicaps caused by some genetic metabolic disorders may never be improved by somatic gene therapy, however successful a gene transfer protocol. Gene transfer is one of the key factors in gene therapy. In this project, we will use gene gun as the tool to transfer human insulin gene into schistosoma, and consider calcium phosphate coprecipitation, laser gene transfer, etc. as the potential candidates.

Much interest has been shown in the use of lasers for nonviral targeted gene transfer, since the spatial characteristics of laser light are quite well defined (Ogura, 2004).

Shirahata et al made a small hole in a cell membrane by pulse laser irradiation to help a gene contained in a medium to be transferred into the cytoplasm through the hole. This hole disappears immediately with the application of laser irradiation of the appropriate power (Shirahata, 2001).

2. Research Design and Methods

Several techniques are currently used to transfer genes into various cells, tissues and organs. Although gene therapy is a potential therapeutic approach for arterial restenosis and angiogenesis, the efficiency of transfection is low regardless of the technique used. To transfer gene efficiently, a novel method laser radiation on gene transfection will be studied in this project. Pulse-wave Nd:YAG laser, Ho:YAG laser, UV excimer laser will be used.

(1) Cell culture: Mouse heart smooth muscle cells will be primarily cultured with a standard technique.
(2) Schistosome: Schistosome will be raised for gene transfection.
(3) Mouse: 100 mice will be housed for the gene transfection.
(4) Gene used: Gene with fluerescence label will be used in the project.
(5) Gene transfection: Gene transfection will be done using laser beam.

As other potential candidate, calcium phosphate coprecipitation method will be considered (Sambrook, 1989; Frederick, 1992; Ausubel, 1992)**:**

(1) **Growth of E. coli:**

Dissolve E. coli in 0.3 ml LB plus tetracycline (2 mg/ml) medium, transfer it into a tube containing 5 ml LB plus tetracycline (2 mg/ml) medium, 37^{o}C overnight, then freeze the E. coli (amplify in several tubes before freeze to get more samples).

(2) **Harvesting E. coli:**

A. Streak an inoculum across one side of a plate using sterile technique. Resterilize an inoculating loop and streak a sample from the first streak across a fresh part of the plate, then incubate at 37^{o}C until colonies appear (overnight).
B. Transfer a single bacterial colony into 2 ml of LB medium containing tetracycline (2 mg/ml) in a loosely capped 15-ml tube. 37^{o}C overnight with vigorous shaking.
C. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at 12,000g for 30 seconds at 4^{o}C in a microfuge. Store the remainder of the culture at 4^{o}C.
D. Remove the medium by aspiration.

(3) Lysis of E. coli:

A. Resuspend E. coli pellet in 100 μl of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0)
B. Add 200 μl of freshly prepared Solution II (0.2 N NaOH, 1% SDS), inverting the tube rapidly 5 times. Do not vortex. Store at 4^{o}C.
C. Add 150 μl ice-cold Solution III (5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, H_2O 28.5 ml), gently vortex, store on ice for 3-5 min.
D. Centrifuge at 12,000g for 5 min, 4^{o}C. Transfer the supernatant to a fresh tube.
E. Add 2 volumes of ethanol, Mix by vortex, keep at room temperature for 2 min.
F. Centrifuge at 12,000g for 5 min at 4^{o}C.
G. Remove supernatant and any drops of fluid adhering to the walls of the tube.
H. Rinse the pellet of DNA with 1 ml of 70% ethanol at 4^{o}C, then remove supernatant and any drops of fluid adhering to the walls of the tube.
I. Redissolve the DNA in 50 μl of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 μg/ml). Vortex briefly. Store at 20^{o}C.

(4) Purification of plasmid:

A. Transfer the DNA solution to a 15-ml Corex tube, and add 3 ml of an ice-cold solution of 5 M LiCl. Mix well, and then centrifuge at 10,000 rpm for 10 min at 4^{o}C.
B. Transfer the supernatant to a fresh 30-ml Corex tube. Add an equal volume of isopropanol. Mix well. Recover the precipitated DNA by centrifugation at 10,000 rpm for 10 min at room temperature.

C. Decant supernatant carefully, and invert the open tube to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the tube with 7% ethanol at room temperature. Drain off the ethanol entirely.
D. Dissolve the pellet in 500 μl of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 μg/ml). Transfer the solution to a microfuge and store at room temperature for 30 min.
E. Add 500 μl of 1.6 M NaCl containing 13% (w/v) polythylene glycol (PEG 800). Mix well. Recover the plasmid DNA by centrifugation at 12,000g for 5 min at 4°C.
F. Remove supernatant. Dissolve the pellet of plasmid DNA in 400 μl of TE (pH 8.0). Extract the solution once with phenol, once with phenol:chloroform, and once with chloroform.
G. Transfer the aqueous phase to a fresh microfuge tube. Add 100 μl of 10 M ammonium acetate, and mix well. Add 2 volumes (~1 ml) of ethanol, and store at room temperature for 10 min. Recover the precipitated plasmid DNA by centrifugation at 12,000g for 5 min at 4°C.
H. Remove the supernatant. Add 200 μl of 70% ethanol at 4°C. Vortex briefly, and then centrifuge at 12,000g for 2 min at 4°C.
I. Remove the supernatant, and store the open tube on the bench until the last visible traces of ethanol have been evaporated.
J. Dissolve the pellet in 500 μl of TE buffer (pH 8.0). Measure the $OD_{260\ nm}$ of a 1:100 dilution (in TE, pH 8.0) of the solution. Calculate the concentration of the plasmid DNA: 1 $OD_{260\ nm}$ = 50 μg of plasmid DNA/ml. Store the DNA in aliquots at -20°C.

(5) Transfer human insulin gene into schistosoma:
A. Gene gun method will be used in the gene transfer.
B. Detect: 12-48 hours after the addition of plasmid, measure the amount of human insulin product with ELISA. The primary antibody used in ELISA is specific active to swine hormone.

(6) Select the male worms with positive human insulin gene.

(7) Infect the male schistosoma transferred with schistosoma into human body.

3. Results

(1) Increased Temperature Enhanced Gene Transfer

The heated cultured human aorta smooth muscle cells had a significantly higher expression of the transfected swine growth hormone gene (Ma, 2004a). Incubated the swine growth hormone gene and human smooth muscle cells under the different incubation temperature of 23°C, 37°C and 43°C, the transfection increased with the temperature elevation ($p<0.01$). The greatest effects occurred within 10 min of incubation and persisted up to 30 min. In another experiment, we got the same result to transfer human interleukin-2 gene into cultured rat myocytes (Ma, 2004b). The results suggest that even a few degrees of ambient temperature rise can significantly increase gene transfer into cells. This may be of value when using gene therapy with transfection procedures.

(2) Laser Enhanced Gene Transfer

UV excimer laser ($XeCl_2$, 308 nm excimer laser, Spectranetics CVS-300™, Spectranetics, Colorado Springs, CO) was done with a 2.0 mm diameter optical fibers. Human aorta smooth muscle cells were cultured in F12K medium containing 2 mM glutamine, 10 mM HEPES, 10 mM TES, 50 ng/ml ascorbic acid, 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml sodium selenite and 30 μg/ml endothelial cell growth supplement, FBS 10% (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA). Gene labeled with anti-amphiline mutation was used. The results showed that laser enhanced the gene transfer.

4. Discussions

Several techniques are currently used to transfer genes into culture cells and into various tissues and organs. These include electroporation, lipofection, calcium phosphate coprecipitation and DEAE-dextran, etc. Although gene therapy is a potential therapeutic approach for arterial restenosis following angioplasty, the efficiency of transfection is low regardless of the vector used. In our primary report it showed that when cultured human aorta smooth muscle cells and endothelial cells and perfused rat aorta artery in a chamber are heated at 45°C or 50°C for up to 1 or 2 hours, transient transfection by calcium

phosphate coprecipitation of a plasmid expressing human growth hormone is enhanced. Transfection of human interleukin-2 gene and swine growth hormone gene into the human smooth muscle cells and rat artery are also enhanced by heating. The results of our study suggest that the relatively low efficiency of gene transfer into tissues for therapy might be increased by short periods of heating during transfection (Ma, 2004a; Ma, 2004b). Also, the gene gun could be a effective technique to practice gene transfer.

5. Summary

The scientific objective of this study is to improve the gene transfer by laser method.

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(8) Clinical trial to the gene therapy.

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