Increased Ambient Temperature Enhances Human Interleukin-2 Gene Transfer into Cultured Rat Myocytes

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Abstract: Background: 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. **Methods:** Rat heart muscle cells were cultured in medium 199 with 10% FBS. Human interleukin-2 gene transfection was performed by calcium phosphate coprecipitation at various temperatures: 23ºC, 37ºC and 43ºC. Interleukin-2 expression was detected using an indirect ELISA. **Results:** The heated cultured rat myocytes had a significantly higher expression of the transfected interleukin-2 gene. Ambient temperature rise to 43°C for up to 30 min provided greater transient transfection of the interleukin-2 gene when compared to ambient temperatures at 37°C and 23°C (p<0.01). The greatest effects occurred within 10 min of incubation and persisted up to 30 min. **Conclusions:** These results suggest that even a few degrees of ambient temperature rise can significantly increase gene transfer into muscle cells. This may be of value when using gene therapy with transfection procedures. [The Journal of American Science. 2005;1(3):51-55]

Key Words: cell; gene transfer; interleukin; rat; temperature

Abbreviations: ATCC, American Type Culture Collection; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF, tumour necrosis factor; CRP, C-reactive protein; MCP, monocyte chemoattractant protein

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 (Selkirk, 2004). Gene transfer is one of the key factors in gene therapy, and it is one of the key purposes of the clone (Ma, 2004). Many serious diseases such as the tragic mental and physical handicaps caused by some genetic metabolic disorders may healed by gene transfer protocol. There is a further implication in that it involves only specific sequences containing relevant genetic information (Bubenik, 2004). 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 (IL) and tumour necrosis factor (TNF), C-reactive protein (CRP), and monocyte chemoattractant protein (MCP) 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.

Materials and Methods
I. Rat Heart Muscle Cells Were Primarily Cultured:
1. Adult rats (about 200 g) were used in this experiment.

2. Rat was sacrificed by decapitation with a decapitator.

3. Rat heart was moved out and left atrium was isolated under sterile controlling.

4. Tissue was transferred to a fresh sterile phosphate buffered solution (PBS) and rinse.

5. Transfer to a second dish and dissect off unwanted tissue such as fat or necrotic material and chop finely with crossed scalpels to about 1 mm cubes.

6. Transfer by pipette (10 – 20 ml with wide tip) to a 15-ml sterile centrifuge tube.

7. Wash by resuspending the pieces in PBS, transfer the chopped pieces to the trypsinization flask, and add 1 ml trypsin solution (0.25%) per 100 mg tissue. Incubate the tissue in trypsin solution for 12 hours at 4°C then wash with PBS for 3 times.

8. Add 1 ml trypsin solution (0.25%) per 100 mg tissue, with 1 mg/ml elastase and 1 mg/ml collagenase then stir at about 200 rpm for 30 min at 36.5^{o}C.

9. Allowing the pieces to settle, collect supernatant, centrifuge at approximately 500 g for 5 min, resuspending pellet in 10 ml medium with 10% serum (FBS) (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA), and store cells on ice.

10. Add fresh trypsin to pieces and continue to stir and incubate for a further 30 min. Repeat steps 6 – 8 until complete disaggregation occurs or until no further disaggregation is apparent.

11. Collect and pool chilled cell suspensions, and count by hemocytometer.

12. Dilute to 10^{6} per ml in growth medium and seed as many flasks as are required with approximately 2 x 10^{5} cells per ml or set up a range of concentrations from about 10 mg tissue per ml.

13. Put into CO_2 incubator with 36.5^{o}C.

14. The culture medium used was Medium 199 with 10% FBS. All the solutions used contain 0.1 mg/ml of anti-biotic ampicillin (Sigma Chemical Co., St Louis, MO, USA).

II. Bacteria Culture (Sambrook, 1989; Frederick, 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°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°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 $-20^{o}C$.

III. Purification of plasmid:

1. 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 $4^{o}C$.

2. 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.

3. 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.

4. 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.

5. 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 $4^{o}C$.

6. 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.

7. 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 $4^{o}C$.

8. Remove the supernatant. Add 200 μl of 70% ethanol at $4^{o}C$. Vortex briefly, and then centrifuge at 12,000 g for 2 min at $4^{o}C$.

9. Remove the supernatant, and store the open tube on the bench until the last visible traces of ethanol have been evaporated.

10. 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 $-20^{o}C$.

IV. Transfer human interleukin-2 gene into rat heart muscle cells:

1. **Transferred gene:** Human interleukin-2 (IL-2) gene cloned in plasmid pBR322 inserted in Escherichia coli (E. coli) were bought from American Type Culture Collection (ATCC, Rockville, MD, USA).

2. **Transfection:** ~2×10^{7} cells suspended in 0.2 ml medium were seeded into a tissue culture chamber. 48-72 hours later, remove

medium and add 0.2 ml fresh medium, then add 0.5 µg of plasmid in 0.05 ml calcium phosphate-HEPES-buffered saline, pH 7.0. Control the chamber temperature at 23°C, 37°C and 43°C separately, or laser treatment. 4 hours (or other time length) later, change medium.

V. Detection of interleukin-2:
12-48 hours after the addition of plasmid and incubation, the amount of interleukin-2 was measured with the indirect enzyme-linked immunosorbent assay (ELISA) in medium. Antibody of anti-interleukin-2 (human) was gotten from Sigma.

Results
Incubated the human interleukin-2 gene with the rat heart muscle cells, it got positive results for the human interleukin-2 gene transferred to rat cells. At the beginning of the incubation, there was the same background for the interleukin-2 measurement (p=ns). The elevated temperature from 23°C to 37°C then to 43°C significant increased the gene transfer product expression (p<0.01) (Figure 1). The greatest effects occurred within 10 min of incubation and persisted up to 30 min. There was significant different interleukin-2 expression for 37°C then to 43°C only after 3 min incubation. 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.
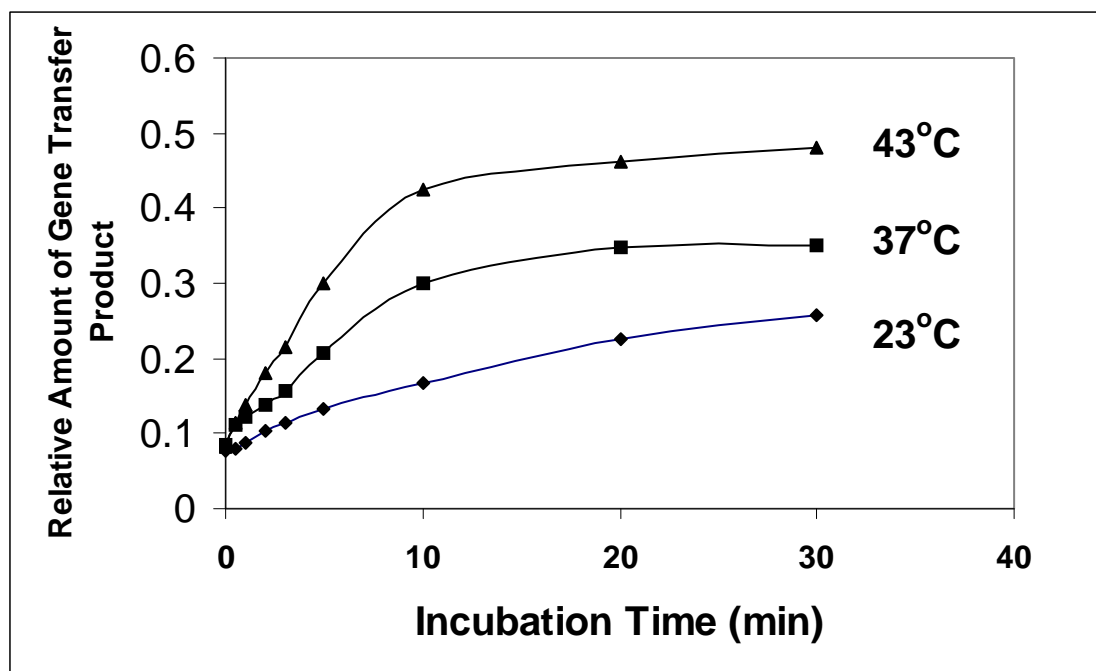

Figure 1. Human Interleukin-2 Gene Transfer under Different Temperature.

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 this experiment our data show 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. Also, the gene gun could be an effective technique to practice gene transfer.

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