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CARDIAC AND PULMONARY REPLACEMENT

EFFICIENCY OF A HIGH-TITER RETROVIRAL VECTOR FOR GENE TRANSFER INTO SKELETAL MYOBLASTS

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Background: Genetic transformation of skeletal myoblasts for myocardial repair is dependent on an efficient gene transfer system that integrates the genes of interest into the genome of the target cell and its progeny. The aim of this investigation was to evaluate the use of a new retrovirally based gene transfer system for this purpose. **Methods:** MFGnslacZ retroviral vector, packaged in high-titer, split-genome packaging cell line (FLYA4) was used to transduce the skeletal myoblast cell line L6. L6 cells, cultured in 10% fetal calf serum, were transduced with the MFGnslacZ vector by means of filtered supernatant from FLYA4 cells. Transduced L6 cells were divided into four groups. Group I cells were fixed as myoblasts 3 days after transduction. Group II cells were allowed to differentiate into myotubes. Group III cells were split every 3 days for 4 months. Group IV cells were split as in group III but then allowed to differentiate into myotubes. All samples were fixed and stained for β -galactosidase activity. The effects on gene transfer of transforming growth factor- β , insulin-like growth factor-I, and platelet-derived growth factor were determined by spectrophotometric assay of β -galactosidase activity in cells transduced in the presence or absence of serum with 0 to 200 ng/ml of each growth factor. **Results:** Morphometric analysis showed that $66.3\% \pm 3\%$ to $69.6\% \pm 6\%$ of cells in

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groups I to IV expressed the *lacZ* reporter gene. In the presence of serum, transforming growth factor- β significantly inhibited gene transfer, whereas insulin-like growth factor-I and platelet-derived growth factor significantly enhanced gene transfer. In absence of serum, however, only platelet-derived growth factor enhanced retrovirally mediated gene transfer into skeletal myoblasts. **Conclusion:** MFG retroviral vectors packaged in FLYA4 cells are efficient in gene transfer into skeletal myoblasts and result in transgenic expression that is maintained after repeated cell division, differentiation, or both. Platelet-derived growth factor enhances retrovirally mediated gene transfer into skeletal myoblasts. (J Thorac Cardiovasc Surg 1998;115:1-8)

Genetic transformation of skeletal myoblasts is an important aim of gene transfer, not only for the treatment of skeletal myopathies¹ but also for myocardial repair.^{2,3} Two main experimental approaches have been adopted for myocardial repair by skeletal muscle cells: direct injection of skeletal myoblasts into the heart^{2,3} and transformation of fibroblasts into skeletal muscle cells by means of the myogenic basic-helix-loop-helix transcription factor MyoD.⁴ Skeletal muscle cells, however, lack essential cardiac-specific proteins, such as gap-junction proteins, that allow direct intercellular communication and act as low-resistance pathways for the propagation of action potential in myocardium.⁵ We are currently exploring the possibility of genetically transforming skeletal myoblasts to express the gap-junction protein (connexin 43). The success of this approach is dependent on establishing a gene-transfer system that allows permanent integration of the "therapeutic" gene into the chromosome of the target cells and their progeny.

Unlike adenoviral vectors, both adeno-associated and retroviral vectors integrate into the chromosome.^{6,7} We chose a retroviral vector for these studies because their biology is well known and they have been in use in clinical gene therapy since 1989 without serious side effects.⁶ Most of these clinical protocols use vectors that are based on the Moloney Murine Leukemia Virus (MoMuLV), which is rendered replication-defective by deletion of sequences coding for essential proteins required for the packaging of the viral particles.^{6,8} The gene of interest is then cloned into this vector, and the resulting construct is reproduced in cells containing the genes required for viral packaging.⁹ Such vectors have been used to carry a dystrophin minigene into skeletal myoblasts *in vitro* and *in vivo*, with an efficiency of 5% to 10%.^{10,11} The low rate of gene transfer in these studies has been attributed to

inadequate viral titer, poor packaging efficiency, and the instability of the vectors produced.

The MFG retroviral vector, which is based on the MoMuLV, has been engineered to allow efficient translation and packaging of recombinant vectors.^{8,12} Furthermore, retroviral packaging cell lines such as FLYA4 produce high-titer retroviral vectors that are more stable than previous vectors.¹³ One limitation of retrovirally mediated gene transfer is the need for the target cells to be actively dividing to allow gene integration.⁴ To circumvent this limitation, growth factors have been used to enhance retrovirally mediated gene transfer into hematopoietic stem cells.¹⁵ In this study, we investigated the efficiency of the MFG retroviral vector, packaged in FLYA4 cells, in gene transfer into the rat skeletal myoblast cell line L6. We also tested the effects of the growth factors insulin-like growth factor-I (IGFI), transforming growth factor- β (TGF- β), and platelet-derived growth factor (PDGF) on gene transfer.

Materials and methods

Cells lines and media. The MFG vector has previously been engineered to carry the *lacZ* reporter gene, which encodes the bacterial enzyme β -galactosidase with a nuclear localization signal (*nls*).^{8,12} The vector was packaged in FLYA4 cells¹³ (gift from Professor Weiss, Chester Beatty Laboratories, Institute for Cancer Research, London). This cell line was derived from the human fibrosarcoma HT1080 cells (American Type Culture Collection, Rockville, Md.) and has been genetically engineered to express the *gag-pol* and the *env* genes on two separate transcription units.¹³ The number of infectious units produced was determined by transducing NIH 3T3 murine fibroblast cell line (American Type Culture Collection) in a serial titration. The skeletal myoblast cell line L6 (American Type Culture Collection) provided target cells for gene transfer. The cells were maintained in 75 cm² filter flasks containing Dulbecco's modified Eagle's medium (Sigma, Poole, United Kingdom) supplemented with 2 mmol/L glutamine, 50 μ mol/ml penicillin, 50

Fig. 1. Estimation of BTE of the MFG vector. Schematic representation of protocol used to study the vector. L6 myoblasts were split in 12-well multiwell plates at a density of 0.5×10^5 cells per well. At 60% confluence, cells were transduced by adding 1 ml medium containing 10^6 infectious units of MFGnlsLacZ. At time 0 (TE_0), the medium in well number 1 was replaced with 1 ml viral supernatant to allow adsorption of vector to the L6 cells. After 1 hour of incubation at 37°C , the medium from well number 1 was completely aspirated and transferred to well number 2 (TE_1) for 1 hour. This procedure was repeated every hour for wells 3 to 6. At time 0, 30 ml viral supernatant was incubated at 37°C in a 50 ml Falcon tube. From this stock, after the first hour, 1 ml viral supernatant was added to well number 7 for 1 hour then replaced with the appropriate medium. The same procedure was repeated hourly for wells 8 to 11. After exposure to the virus, the cells were maintained in 10% FCS or serum-free medium for 48 hours. The cells were then fixed and stained for β -galactosidase activity. The number of cells successfully transduced with *lacZ* was determined by morphometric analysis. BTE was determined from the formula $BTE = TE_0 / (TE_0 - TE_1)$. To determine the influence of serum withdrawal on gene transfer, we calculated BTE with the formula $BTE = TE_{0A} / (TE_{0A} - TE_{0B})$, where TE_{0A} and TE_{0B} are the transduction efficiencies measured without previous adsorption in 10% FCS (first hour) and without adsorption in serum-free medium (first hour), respectively.

$\mu\text{mol/ml}$ streptomycin, and 10% fetal calf serum (FCS). Serum-free medium consisted of OPTIMEM 1 (Sigma) supplemented with 50 $\mu\text{mol/ml}$ penicillin and 50 $\mu\text{mol/ml}$ streptomycin. All cells were incubated at 37°C in a humidified chamber equilibrated with 5% (volume/volume) carbon dioxide in air.

Gene transfer into skeletal myoblasts and determination of MFG transduction efficiency. To determine the ideal concentration of viral vectors in transducing skeletal myoblasts, L6 cells were plated in 6-well multiwell plates at a density of 1×10^5 cells/well. At 60% confluence, cells were transduced by adding 4 ml medium containing either 10^6 , 10^5 , 10^4 , 10^3 , or 10^2 infectious units and reincubated for 3 days. The cells were then fixed and stained for β -galactosidase activity and subjected to morphometric analysis. We then estimated the biologic transduction efficiency (BTE) of the MFG vector, as described by Tavoloni¹⁶ and summarized in Fig. 1. In these and the following experiments, we used

10^6 infectious units/ml. The target cells were exposed to only one dose of viral supernatant for 3 days unless otherwise indicated.

Another group of L6 cells was cultured in 25 cm^2 tissue culture flasks, at a density of 1×10^6 cells/flask, and incubated as outlined previously. At 60% confluency, L6 cells were transduced by adding MFGnlsLacZ to the concentration of 10^6 infectious units/ml. Transduced L6 cells were then divided into the following four groups:

Group I: Cells were fixed as myoblasts 3 days after transduction and were then stained.

Group II: Cells were allowed to differentiate into myotubes by reducing serum concentration to 2% for 7 days and were then fixed and stained.

Group III: Cells were split by trypsinization every 3 days (1:4), thereby maintaining them as dividing myoblasts, for 4 months and were then fixed and stained.

Group IV: Cells were split as in group III but allowed to

Fig. 2. L6 skeletal myoblasts transduced with either 10^6 , 10^5 , 10^4 , 10^3 , 10, or 0 viral particles/ml (panels 1, 2, 3, 4, 5, and 6, respectively). Panel 6 is the untreated (negative) control.

differentiate into myotubes in medium containing 2% FCS and were then fixed and stained.

For each group, untransduced L6 cells were used as a control.

Staining for β -galactosidase activity and morphometric analysis. After fixation in 3.7% formaldehyde for 1 hour, L6 cells were washed and stained for β -galactosidase activity by adding 1 ml of X-Gal staining solution (Sigma) for 2 to 6 hours at 37° C. For morphometric analysis, each flask was overlaid by a transparent grid consisting of squares 1×1 mm, and each 1 mm square in the field of view was placed in register with the square grid of an eyepiece graticule of a light microscope (Zeiss, 25FL, Welwyn Garden City, United Kingdom) that contained 121 intersections. Sampling started in the upper left part

of the section. Every fourth 1 mm square was selected for point counting. For each selected square, the number of intersections falling on cells with stained nuclei was recorded. The mean values of six readings from each sample were recorded.

Effects of growth factors on gene transfer into skeletal myoblasts. The L6 cells were seeded in 24-well multiwell plates at 0.5×10^5 cells/well in 10% FCS. After 16 hours, the medium was replaced by serum-free medium for 6 hours before addition of filtered viral supernatant containing IGFI, TGF- β , or PDGF(BB) at 0 to 200 ng/ml. Cells were then maintained in serum-free or 10% FCS-containing medium. After 72 hours, all cells were lysed by adding 100 μ l reporter lysis buffer (Promega, Southampton, United Kingdom). β -Galactosidase activity was de-

Fig. 3. Transduced L6 cells were divided into four groups. Group I cells were fixed as myoblasts 3 days after transduction then stained (**A**). Group II cells were allowed to differentiate into myotubes by reducing serum concentration to 2% for 7 days were then fixed and stained (**B**). Group III cells were split (1:4) every 3 days for 4 months and were then fixed and stained (**C**). Group IV cells were split as in group III but were allowed to differentiate into myotubes in 2% FCS and then were fixed and stained (**D**).

terminated by incubating 10 μ l cell lysate with 200 μ l Z-buffer containing *o*-nitrophenyl β -D-galactopyranside (ONPG; Sigma) for 4 to 8 hours.¹⁷ The optical density (OD) was then measured spectrophotometrically at 414 nm and corrected for total protein contents. β -Galactosidase activity, expressed as OD units per microliter per hour, was calculated according to the following formula:

$$\text{OD units}/\mu\text{l/hr} = A_{420} \times 1000/(\mu\text{l extract used} \\ \times \text{incubation time in hours})$$

Statistics. Data are presented as mean $\pm$ standard error of four samples. Student's *t* test was used to compare test samples to controls. A *p* value less than 0.05 was considered significant.

Results

The number of L6 cells transduced with MFGnlsLacZ was dependent on the number of infectious units added to the medium (Fig. 2).

Fig. 4. Retroviral BTE (TE) in the presence (FCS) or absence (SFM) of serum, tested as described in Fig. 1.

Table I. Effects of growth factors on gene transfer into skeletal myoblasts

	β -Galactosidase activity after transduction in 10% FCS (OD units/ μ g/hr)	β -Galactosidase activity after transduction in SFM (OD units/ μ g/hr)
Control (no growth factors)	7.1 $\pm$ 0.7	2.0 $\pm$ 0.2
IGFI (20 ng/ml)	10.6 $\pm$ 0.4, $p = 0.02$	2.6 $\pm$ 0.2, $p = 0.059$
TGF- β (200 ng/ml)	2.7 $\pm$ 0.1, $p = 0.008$	2.2 $\pm$ 0.3, $p = 0.142$
PDGF (100 ng/ml)	11.4 $\pm$ 0.8, $p = 0.005$	4.5 $\pm$ 0.3, $p = 0.039$

L6 cells transduced with MFnslacZG in presence or absence of 10% FCS with or without IGFI, TGF- β , or PDGF(BB). β -Galactosidase activity was assayed spectrophotometrically. Data presented as the mean value of four experiments $\pm$ standard error. SFM, Serum-free medium.

Transduction of $65.0\% \pm 6\%$ of cells was achieved with 1×10^6 viral particles/ml. There was no significant gene transfer with concentrations less than 1×10^4 viral particles/ml. β -Galactosidase was not expressed in the control groups. In groups I, II, III, and IV, the percentages of cells expressing the transgene were $66.3\% \pm 3\%$, $69.6\% \pm 6\%$, $68.9\% \pm 5\%$, and $67.4\% \pm 4\%$, respectively (Fig. 3). There was no significant difference in the proportion of cells expressing the reporter gene *lacZ* in cells stained before and after

repeated cell division, differentiation, or both. Because the cell-cycle duration of L6 cells is around 18 hours, we estimate that cells in groups III and IV would have undergone a minimum of 160 cell divisions during a period of 4 months.

The BTE of the MFG vector, defined as the number of infectious units present in 1 ml vector stock solution capable of transducing L6 cells, was 76 units/ml/hr in the presence of serum and 52 units/ml/hr in the absence of serum. The rate of viral decay varied from 5% to 23% per hour in cells maintained in 10% FCS and from 5% to 44% per hour in cells maintained in serum-free medium (Fig. 4). In these experiments, the cells were exposed to the virus for 1 hour only, thus the lower transduction efficiency compared with cells exposed for 3 days.

The maximum β -Galactosidase activities observed with each growth factor in cells cultured in 10% FCS-containing or serum-free medium are presented in Table I. In the presence of serum and TGF- β (200 ng/ml), β -Galactosidase activity was reduced from 7.1 ± 0.7 to 2.7 ± 0.1 OD units/ μ l/hr ($p = 0.008$). By contrast, IGFI (20 ng/ml) and PDGF (100 ng/ml) increased β -Galactosidase activity to 10.6 ± 0.4 and 11.4 ± 0.8 OD units/ μ l/hr, respectively. In absence of serum, however, the inhibitory

effect of TGF- β and the stimulatory effect of IGFI did not reach statistical significance. The positive influence of PDGF on gene transfer into skeletal myoblasts, with and without serum, were dose and time dependent (data not shown).

Discussion

Cell transplantation is a potential therapy for chronic myocardial disease.^{2,3} Although transplanted neonatal cardiac myocytes can functionally integrate with and even augment the function of the recipient heart,¹⁸ this approach is limited by shortage of donors and chronic graft rejection. In contrast, skeletal myoblasts—present in adult skeletal muscle as satellite cells—are available in abundance and can be grafted, successfully, into the subject's own heart even after manipulation *ex vivo*.^{2,3} Functional integration of these cells, however, may be hampered by the lack of intercellular gap-junction communication and the variations of excitation-contraction coupling between skeletal and cardiac myocytes.^{5,19} In studies aiming at genetic transformation of skeletal muscle cells to exhibit gap junctions and cardiac-type excitation-contraction coupling, we assessed the use of the MFG retroviral vector in transferring the reporter gene *lacZ* into skeletal myoblasts.

We found that an MFG-based, amphotropic, retroviral vector induces stable gene expression in $66.3\% \pm 3\%$ to $69.6\% \pm 6\%$ of skeletal myoblasts and myotubes. There was no obvious deleterious effect of genetic transformation on the growth rate of L6 cells or on their ability to differentiate into multinucleated myotubes. Transduced L6 cells have not been followed up for longer than 4 months. Previous *in vitro* studies, with earlier generations of recombinant retroviral vectors, showed that only 5% to 10% of skeletal myoblasts can be transduced.^{10,11} The higher rate of successful gene transfer observed in this study may be attributed to two main factors. First, we used a retroviral packaging cell line that produces a high titer of stable retroviral vectors.¹³ The FLYA4 packaging cell line produced 10^6 to 10^7 infectious units/ml. The vectors produced were stable for as long as 6 hours if used to transduce cells cultured in 10% FCS. These findings are consistent with those reported by Cosset and associates,¹³ who found that 50% to 90% of MFGnslacZ, packaged in FLYA4 cells, was stable in human serum for 1 hour. Second, the use of the MFG retroviral vector, in which a small fragment containing the 5'

untranslated region of the MoMuLV envelope gene was inserted downstream of the *gag* sequence ($\psi+$) and upstream of the complementary deoxyribonucleic acid insertion site. These changes result in an increase in synthesis of the spliced ribonucleic acid, which is likely to be translated with greater efficiency than is unspliced ribonucleic acid because of excision of the ψ region.^{12,20} Recent studies have shown that MFG-based vectors are seven times more efficient in gene transfer than are standard retroviral vectors.²⁰

The BTEs of retroviral vectors are dependent on the vector used, the cell type, and the tissue-culture conditions.⁶ We observed significant reduction in the BTE of the MFG/FLYA4 gene transfer system on withdrawal of serum. This observation is consistent with the fact that retroviral integration is dependent on mitosis¹⁴ and that the lack of serum triggers withdrawal of skeletal myoblasts from the cell cycle. Poor retroviral transduction in the absence of FCS mirrors the low rates of *in vivo* retrovirally mediated gene transfer into skeletal muscle where only a few cells are actively dividing.^{11,12}

Growth factors are known to enhance retrovirally mediated gene transfer into hematopoietic stem cells, where the use of basic fibroblast growth factor in combination with interleukin-3, interleukin-6, and stem-cell factor increases the BTE by 35% to 37%.¹⁵ In this study, we found PDGF(BB) and IGFI to be significant enhancers of retrovirally mediated gene transfer into skeletal myoblasts. In contrast, TGF- β in high concentrations had a significant inhibitory effect on gene transfer. The mechanism for this has not been studied; however, PDGF and IGFI are known to stimulate L6 proliferation.^{21,22} Furthermore, TGF- β has been shown to inhibit proliferation of L6 cells.²³ On the basis of this evidence only, it seems that the effect of these growth factors on the rate of cell division may be the main mechanism for enhancement or retardation of gene transfer. Other mechanisms, however, such as increased affinity of retrovirus-specific surface receptors, may also be involved.

In summary, recombinant retroviral vectors packaged in high-titer, split-genome packaging cells are efficient in gene transfer into skeletal myoblasts and result in transgenic expression, which is maintained even after repeated cell division, differentiation, or both. PDGF enhances retrovirally mediated gene transfer into skeletal myoblasts in the presence or absence of serum. These findings may have impor-

tant implications with respect to the clinical use of genetically modified skeletal muscle cells for cardiac assistance.

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Discussion

Dr. Ray C. J. Chiu (*Montreal, Quebec, Canada*). It's a nice and useful study. So far your studies have been in vitro. Do you think your retroviral vector might express antigenicity in vivo?

Mr. El Oakley. Retroviral vectors are not known to express any antigenicity, but they cannot transduce nondividing cells. So if you intend to use retroviral vector to transform a whole adult muscle, you will not be able to do that easily. However, if you intend to take the skeletal satellite cells, as you've done in some of your studies, you could transform the myoblasts as myoblasts and inject them back into the diseased myocardium.

Dr. King F. Kwong (*St. Louis, Mo.*). I have two questions. First, regarding your vector, what is the maximum gene insert capacity that your vector can accommodate?

Second, connexin 43 is the principal cardiac connexin that is already widely expressed in both the atria and ventricle. By transfecting the heart with a connexin 43 gene construct, what do you hope to achieve?

Mr. El Oakley. The maximum gene insert capacity is about 8 kb. What we hope to do in the future is to take skeletal myoblast biopsy samples, isolate the satellite cells, transduce them to express gap junctions, and then inject these cells back into the diseased myocardium. We hope that these cells would integrate with the surrounding myocardium, thereby enhancing cardiac function.

Dr. D. Glenn Pennington (*Winston-Salem, N.C.*). At the most recent meeting of the International Society for Heart and Lung Transplantation, there was some indication that one could increase the number of cells transfected by doing it at higher atmospheric pressure, for example, 2 atm. Have you tried that?

Mr. El Oakley. No, we haven't tried that.