ENHANCED MYOBLAST MEDIATED GENE TRANSFER INTO MYOCYTES FOR THE PRODUCTION OF THERAPEUTIC PROTEINS

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Abstract: The major limitation of myoblast mediated gene therapy has been the low incorporation efficiency. We have identified that partially purified aqueous muscle extracts of rats, which was supposed to contain myoblast specific fusion factor(s) (MSF), could significantly enhance the incorporation rate of genetically engineered myoblasts into intact muscle fibers of the host rats. Once incorporated, the nuclei from the donor's myoblasts could express the desired protein(s) in the hybrid muscle fibers. The present study used the strategy of MSF to enhance the fusion of genetically manipulated donor myoblasts, which contained a plasmid carrying BMP-4 coding sequence, with intact muscle. By tracing the fate of donor myoblasts using BrdU labeling, and detecting the expression of BMP-4 in the hybrid myofibers, it demonstrated that in the presence of MSF, numerous BrdU positive nuclei could be seen and the expression of BMP-4 could also be observed in the hybrid fibers of the recipient. While in the control group using BSA to replace MSF, there were only a few BrdU positive signals. This strategy of enhanced myoblast mediated gene transfer would circumvent the obstacle in the current practice of normal or engineered myoblast transplantation in the management of genetic muscle diseases or systemic genetic disorders.

Introduction

In recent years, one of the most interesting findings is that skeletal muscle fibers can be used as recombinant protein factories to produce and secrete proteins, which can act either locally in muscle or systemically, such as Duchenne muscular dystrophy (DMD), diabetics, and etc. [1-2]. Skeletal muscles-the largest organ in the body are easy to access and could accommodate therapeutic genes. Successful introduction of therapeutic genes into muscle fibers could make the muscle act as an autologous secretion organ of therapeutic proteins. It was reported that after fusion with the host muscles, the genetically engineered myoblasts could maintain the over expression of the desired protein without immunosuppression for up to two years—the life span of a mouse [2].

However, the extreme low efficiency of incorporating donor myoblasts into host muscle fibers has long been the major set-back. The efficiency of gene transfer is affected not only by gene delivery, but also by cellular controls on gene expression [3]. Previous animal experiments and clinical trials demonstrated a very poor incorporation of the injected healthy myoblasts into muscle fibers of the recipients [4]. Also in some cases, the desired protein was only expressed at the site of injection [5]. These disappointing results might be due to the following reasons: (I) Myoblasts are equivalent to satellite cells persisting at quiescent stage and they need to be activated to fuse with muscle fibers. (II) There might have some unknown pre-existing myoblast specific fusion factor(s) (MSF) contained in muscle fibers and the tiny injury caused by injection procedure might activate the quiescent myoblasts in close vicinity and allow some of them to fuse with donor myoblasts at injury site as seen in many previous clinical trials and animal experiments [1, 2, 4]. (III) However, the amount of the released MSF through the tiny injection hole was too limited to reach out, and the injected myoblasts outside the immediate site of injection were therefore hardly incorporated into muscle fibers. Based on the above phenomenon, we postulated that a very limited amount of MSF was released from the tiny injury caused by the injection procedure that activated the myoblasts at immediate site of the injection and committed a limited number of the activated donor myoblasts to fuse with the muscle fibers in close vicinity where they could reach. To test our hypothesis and break the major barrier of very limited fusion rate in myoblast mediated gene transfer, we used partially purified muscle extract (PME) containing MSF to enhance the fusion rate of the donor myoblasts, and therefore more efficient gene delivery. We believed that successful application of this enhanced myoblast-mediated gene transfer strategy would lead to an effective therapy for a variety of genetic and acquired diseases, many of which are incurable with current treatment modalities.

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Materials and Methods

*Marker gene, BMP-4, cDNA cloning***:** The full length cDNA coding for bone morphogenetic protein 4 (BMP-4) was obtained by PCR using full length human BMP-4 cDNA as template and oligonucleotide primers corresponding to the N-terminus of BMP-4 with restriction site for *Xbal*, and thus a start codon, preceded the N-terminal coding sequence, and to the C-terminus of BMP-4 with restriction site for *EcoR1*, preceded by a termination codon. This construct was inserted in the *Xbal/EcoR1*sites of a mammalian expression vector (pCI-neo) to create the construct of pCI-neo-BMP-4. The produced pCI-neo-BMP-4 recombinant plasmid was then transformed into NovaBlue cells. The recombinant plasmid DNA was prepared from several transformants and checked by restriction digest mapping and DNA sequencing. After verification of the construct of pCI-neo-BMP-4, the pCI-neo-BMP4 plasmids were then purified.

*Cell culture and transfection***:** Myoblast (C2C12) was grown in Dubecco's modified Eagle's medium (DMEM) containing 5.5mmol/l glucose, 2 mM L-glutamine, 10 % fetal calf serum (FBS) supplemented with 10 % PSN antibiotic mixture. It was incubated at 37 °C in 5 % CO_2-controled water-jacketed incubator with routine culturing procedures. Before transfection, 1×10^4 cells were plated out on 60 mm culture dish. When they reach 50 % confluent, they were either transfected with blank pCI-neo or pCI-neo-BMP-4 recombinant plasmid using FuGENE™ 6 Transfection Reagent. A day after transfection, cells were selected by incubating with 800 μg/ml antibiotic G-418 sulfate containing medium for a week. Then several clones were isolated respectively. The expression of marker gene (BMP-4) of the C2C12 transfectants was confirmed by immunocytochemistry with antibody specific against human BMP-4 and a fluorescein (FITC)-conjugated secondary antibody.

Alkaline phosphatase (ALP) activity and osteocalcin production assay: The transfected myoblasts were seeded onto 60 mm culture dish in DMEM medium containing 10% FBS. When they reach 80% confluence, the culture medium was aspirated and the cells were rinsed with PBS for several times. 500 μl lysis buffer (50 mM Tris-HCl and 0.1 % Triton X-100, pH7.5) was added and cells were scraped from the dish. The cells were then homogenized for 100 times on ice. After homogenization, the cell lysate was collected and centrifuged at 12,000 rpm for 10 minutes. In the case of muscle tissue used, aqueous hybrid muscle extract was prepared. Fifty microliter of supernatant of the cell lysate or aqueous hybrid muscle extract was incubated with 150 μl of p-nitrophenyl phosphate (pNpp) buffer (Metra Biosystems, Inc.) at 37 °C for an hour. The reaction was stopped by adding 100 μl 1 M NaOH. The absorbance at 410 nm was measured using NaOH as blank. The amount of p-nitrophenol (pNp) produced was calculated from a standard curve, which is prepared by using pNp. The amount of protein in cell lysate was measured using Bradford protein assay. The activity of alkaline phosphatase was expressed as micromole of pNp produced per minute per milligram of protein.

The amount of osteocalcin was determined using Osteocalcin ELISA (DAKO) assay kit. Briefly, 50 μl of supernatant of the cell lysate or aqueous hybrid muscle extract was first mixed with 200 μl of Biotinylated osteocalcin for 3 minutes with moderate shaking at 200 rpm. 200 μl of mixture was transferred to 96-wells strips, which were previously been coated with anti-osteocalcin antibody and then incubated for 1 hour with shaking. The strips were washed 5 times with washing buffer. After removing the entire residue in the wells, 200 μl of peroxidase conjugate was added and incubated for 15 minutes with shaking. After extensive washing as before, 200 μl of chromogenic substrate was added and incubated in dark for 30 minutes with shaking again. Finally, the reaction was stopped by adding 50 μl 2 M H_2SO_4. Absorbance was measured at 450 nm by means of spectrophotometry after 2 minutes incubation.

Preparation of the partially purified muscle extract (PME): Leg red (soleus, 91%) muscles of 250 g adult rat were isolated using the procedure described by Bischoff and Li [6, 7]. After isolation, the muscles were transferred to phosphate-buffered saline (PBS) and then crushed with forceps into small pieces. The crushed muscles were incubated in PBS at 4 °C for 2 hours with gentle shaking. After centrifugation at 20,000 rpm for an hour, the supernatant containing MSF was collected. The production of active PME was conducted by following Bio-assay-guided-isolation and protein refolding procedures used in our previous studies [7].

Activation of myoblasts and myoblast labeling: The transfected myoblasts containing pCI-neo-BMP-4 plasmid were plated on culture dish and incubated in 10% FBS containing medium for 2 days. When they reach 50% confluent, medium was removed and replaced with the active PME produced from above procedure for activation. Cells were further cultured for 2 days and the morphologies of cells were observed using phase-contrast light microscopy with magnification of 200× (Olympus).

The activated cells (1×10^4 cells) were plated on 60 mm culture dish. When they reached 50% confluent, the medium was removed and replaced with the medium containing an appropriate amount of BrdU. In order to test the labeling efficiency by this method in cell culture, the myoblasts were fixed in FixDenat and then incubated with anti-BrdU-POD solution for 90 minutes at room temperature. The excessive solution was washed thoroughly with washing solution. Finally, cells were incubated with appropriate amount of substrate solution at room temperature for about 30 minutes until color development is sufficient for photometric detection. Cells were further cultured for 60 hours and the suspension of Pronase-dissociated labeled cells was concentrated by centrifugation. The yield of the labeled myoblasts after culture was $2.0 \pm 0.5 \times 10^6$. The yield of the myoblasts was equivalent to an increase of about 8

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fold. The yield of BrdU labeled myoblasts was near 90% of the total cell.

PME induced massive fusion of donor myoblasts with muscle fibers of recipients: Two groups of 25 female SD rats (weight about 150 g), randomly assigned, and were tested according to the international experimental animal standard [8]. The BrdU labeled PME-pretreated myoblasts (1×10^6) were injected into tibialis anterior muscles of group one rats together with 3 mg PME. For group two rats, the same amount of BrdU labeled myoblasts was injected into the same site together with 1 mg bovine serum albumin (BSA). The experimental rats were sacrificed on 1, 3, 5, 7, 9 days after the injections, respectively. The muscles of tibialis anterior were removed and processed for paraffin sectioning. Immunohistochemistry using anti-BrdU antibody was carried out for tracing the fate of the transplanted donor myoblasts and using anti-BMP-4 antibody for detecting the performance of the introduced foreign marker gene.

Results

Enhanced incorporation of donor myoblasts into host muscle fibers: After verification of the proper expression of BMP-4 polypeptide in the labeled C2C12 transfectants, the labeled PME-pretreated C2C12 transfectants were injected into tibias muscles together with 3 mg PME solution or equivalent BSA respectively. The process of incorporation of donor myoblasts into host muscle fibers was monitored by tracing the fate of the injected BrdU-labeled myoblasts using immunohistochemistry with monoclonal antibody specific for BrdU. It was shown that numerous BrdU positive nuclei were observed in the whole area of the hybrid muscle fibers of the recipient rats (Fig. 1A) when the donor myoblasts were coinjected with PME. In contrast, much less or almost no Brdu-positive nuclei could be observed in BSA control group (Fig. 1B). To confirm whether the fusion of the donor myoblasts with recipient muscle fibers would cause the introduction of pCI-neo-BMP4 plasmid and whether the introduced recombinant plasmid could direct protein synthesis properly in the hybrid myofibers by using the molecular machinery of the host, antibody specific for BMP-4 polypeptide was used to determine the expression of the introduced foreign gene. The positive anti-BMP-4 immunohistochemical staining indicated that BMP-4 polypeptide was translated from the introduced gene construct (Fig.1C) in the hybrid muscle fibers. In contrast, no positive BMP-4 signal was detected in the blank pCI-neo plasmid control (Fig.1D).

Since the nascent polypeptide of BMP-4 and full length BMP-4 exist within intra-cellular space, and once they are exported to the extra-cellular space, the signal peptide and N-terminal pro-polypeptide would be removed to produce mature domain of BMP-4 (MDBMP-4), the antibodies specifically against N-terminal domain of BMP-4 would only recognize the nascent polypeptides and full length of BMP-4 that located within the intra-cellular space (Fig. 1C).

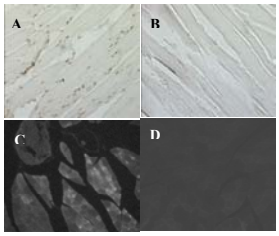

Figure 1: Incorporation of donor myoblasts into host muscle fibers.

BMP-4 induced morphological changes and osteocalcin production in the hybrid muscle fibers: To confirm whether the BMP-4 polypeptide produced by the introduced foreign gene in the hybrid muscle fibers adopted proper folding, trafficking and post-translational modification to produce active mature BMP-4, both morphological and biochemical studies (ALP activity and production of osteocalcin) on the hybrid muscles were performed as did on the C2C12 transfectants during the procedure for verification in cellular level. After one week of the myoblast injection, the hybrid muscle fibers displayed a hardening appearance with stiff boundaries between the neighboring hybrid muscle fibers compared with the control muscle fibers containing blank pCI-neo plasmid (Fig. 1A & Fig. 2). Biochemical assays for the presence of osteocalcin also demonstrated that significant amount of osteocalcin was produced in the hybrid muscle fibers from day 3 and even higher on day 5, 7 and 9 after injection (Fig. 3) suggesting the osteo-differentiation of the hybrid muscle fibers due to the effect of active BMP-4. In contrast, the extract of muscle fibers containing blank pCI-neo plasmid showed no any positive signal of osteocalcin production.

Figure 2: Morphological and biochemical changes of the hybrid muscle fibers.

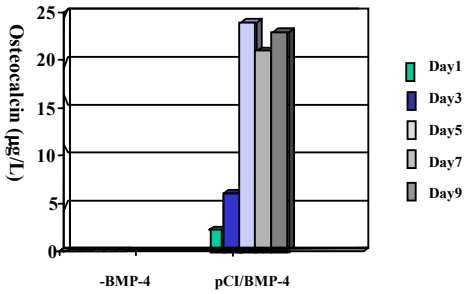

Figure 3: Biochemical assay for the presence of bone marker-osteocalcin.

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Alkaline phosphatase (ALP) activity and biochemical changes: To test whether the expressed BMP-4 polypeptide in the hybrid muscle fibers is active, the activity of alkaline phosphatase (ALP) was also determined. It is well documented that the presence of mature domain of BMP-4 in extracellular space could induce the conversion of myoblast into osteoblast lineage so that ALP- a terminal differentiation marker of osteoblasts would be produced [11, 12]. The results showed that the extract prepared from the hybrid muscle fibers did express BMP-4 polypeptide (Fig. 1C) and displayed significantly higher ALP activity compared with the extract from the hybrid muscle fibers with empty plasmid (pCI-neo) alone or from non-transfectants (Fig. 4).

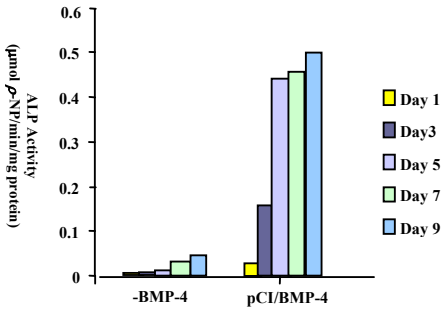

Figure 4: Assay for alkaline phosphatase (ALP) activity

Discussion

In the past decade, gene therapy has been used to treat central nervous system tumors, leukemia, vascular disease, etc [9-11]. However, big challenges to gene therapy remain to be resolved for achieving better clinical results, such as how to efficiently deliver the desired therapeutic gene into a sufficient number of host cells to achieve significant clinical efficacy. Although many efforts have been made, the inefficient fusion of the donor myoblasts with host muscle fibers remains problematic greatly limiting the application of myoblast-mediated gene therapy. Myoblast-mediated gene delivery, despite the unsatisfactory results from clinical trials for the treatment of Duchenne muscular dystrophy (DMD) [12], is still a potential method for the rectification of primary muscle myopathies and some systemic disorders, provided the efficiency of gene delivery can be significantly improved. Previous studies on myoblasts transplantation showed that the injected donor myoblasts could only disperse from the injecting site over a minute distance of a few millimeters, therefore multi-injection protocol was adopted. This phenomenon was well reflected in the clinical trials of DMD, in that the uptake of injected myoblasts into the host muscle fibers was ineffective and disappointing. The majority of injected myoblasts died and was rapidly removed following injection, which was believed to account for the low incorporation rates of donor myoblasts [13, 14]. Our results, in the present study, were consistent with this speculation, in that the injection of labeled myoblasts alone or co-injection of labeled myoblasts with BSA resulted in little incorporation to the muscle fibers of the recipient rats.

From our previous studies and the literature [15-19], we noticed that the procedure of injection caused a tiny injury to the muscle fiber that allowed local release of some intrinsic factors normally enclosed in the muscle fibers. The released factors might trigger the activation and fusion of the transplanted myoblasts with recipient muscle fibers at the site of injection and its immediate vicinity. In consideration of the poor incorporation of the transplanted donor myoblasts in myobalst-mediated gene transfer based clinical trials, we postulated that multi-injections of myoblasts themselves induced multi-tiny point injuries to the muscle fibers of recipients, which lead to the release of wound fluid containing trace amount of MSF. However, the released trace amount of MSF could only sufficiently support a few incorporations of myoblasts at the immediate sites of injections, or their close vicinities. When the released factors diffused beyond those small areas around the injection sites, the concentration of MSF would be too low to support any more incorporation of the transplanted myoblasts. This hypothesis could explain the facts of only a few myoblast incorporation in previous disappointing clinical trials of myoblast mediated gene transfer [4, 5, 20, 21]. This hypothesis formed the ground of the present study. In practice, we cannot make puncture to every muscle fiber due to the technical impossibility and the considerable damage to the patient. Therefore, an alternative procedure was used that could provide sufficient amount of MSF factors along with the donor myoblasts over the desired areas but does not cause much damage to the muscle fibers of the recipient. In this study, donor myoblasts were initially activated ex vivo using PME containing MSF and then coinjected with PME into muscle mass of the recipient. The coinjected MSF would diffuse with the myoblasts along interstitial tissue to most muscle fibers in a reasonable concentration gradient and activate the myoblasts to migrate to the muscle fibers. The efficient fusion of the activated myoblasts with muscle fibers induced by MSF containing fraction would then be attained.

The promising result of significantly increased incorporation of donor myobalsts into recipient muscles demonstrated the consistency with our above hypothesis that there is protein factor(s) (MSF) enclosed in muscle cells that could activate quiescent myoblasts and enhance their migration and fusion with host muscles to form the hybrid muscle fibers. The spectra of the location from central to peripheral regions of the BrdU positive nuclei in the hybrid muscle fibers reflected the different stages of myoblast fusion suggesting the developmental process from early to the maturation stages. Our strategy of MSF enhanced migration and incorporation of donor myoblasts could successfully complete nuclei transplantation of donor myoblasts or delivery of desired gene constructs into host muscle fibers in a controlled manner by adjusting the amount of

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the coinjected MSF. The genetic defects of muscle or certain systemic defects could be corrected by introduction and expression of the normal nuclei or introduced therapeutic gene.

Skeletal muscles can be used as recombinant protein factories that could provide ideal microenvironment and cellular machinery for production, correct folding, molecular trafficking and post-translational modification or secreting of therapeutic proteins that could act either locally in muscle for the treatment of muscular disorders or distally for other systemic disorder [22-25]. The results of this research demonstrated that the introduction of BMP-4 coding sequence into the hybrid muscle fibers could induce the expression of BMP-4 polypeptide. The elevated activity of ALP and the production of osteocalcin (the osteo-differentiation markers) in the hybrid muscle fibers indicated that the produced foreign BMP-4 polypeptide could fold correctly, be efficiently transported into extra-cellular space, and be properly modified to produce active mature domain of BMP-4 in the hybrid muscle fibers.

Skeletal muscle constitutes about 30% of total human body mass and is easily accessible for the delivery of nearly all gene transfection approaches. The abundant blood supply makes skeletal muscles possible for the efficient delivery of the therapeutic proteins into circulation if required. Because the myofibers are post-mitotic and long-lived, they provide a stable environment for continuous production of the introduced therapeutic proteins. In the present study, the conversion of the hybrid muscle fibers into a hardening appearance, and the increased ALP activity and the increased production of osteocalcin in the hybrid muscle fibers, both of which are the positive markers of bone formation, collectively indicated that skeletal muscles can be used as an ideal location for production of active therapeutic protein.

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