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Original Research

Lentiviral mediated overexpression of Insulin like Growth Factor-1 in mouse myoblast

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Abstract: Insulin-like growth factor gene (IGF-1) is one of the most important growth factors that plays a key role in the proliferation and differentiation of muscle cells. IGF-1 is also a radial stimulant in muscle hypertrophy in mammals. In our study, we constructed a lentiviral vector inducing an overexpression of IGF-1 in order to study the regulating mechanisms of this gene. The IGF-1 gene was cloned into the lentiviral shuttle plasmid pCDH-cGFP and the recombinant lentiviral vector was transducted into myoblast C2C12 cell line. The overexpression of IGF-1 was confirmed by RT-PCR and western blotting for IGF-1 receptor gene. Additionally, chemiluminescence results also showed that the concentration of IGF-1 in the transduced cells significantly increased compared to the control group. The results of our study suggests that constructed recombinant lentiviral vector can potentially be used for regulating the expression of IGF-1 in myoblast C2C12 cells.

Key words: IGF-1, overexpression, recombinant lentiviral, gene therapy.

Introduction

Muscle is a highly plastic tissue able to adapt to changing functional demands. Increased load on muscle results in an increase in its mass or hypertrophy(1). Muscle growth in animals can occur by an increase in number and size of myofibrils. Increase in the number of myofibrils is as a result of proliferation and differentiation of satellite cell like C2C12 ,and the myofibril size is influenced by protein synthesis and protein degradation (2).

There is substantial evidence from both *in vivo* and *in vitro* studies demonstrating that IGF-1 is a major regulator of muscle mass during development (3). The function of IGF-1 is also regulated by cross-talk between IGF1R and multiple binding proteins and exerts all of its known physiologic effects by binding to the IGF1R(4).

Regarding the role of IGF-1 in muscle growth, several studies have indicates that IGF-1 can induce hypertrophy and block atrophy. Local infusion of recombinant IGF-1 results in muscle hypertrophy in rats (5). In adult mice, virus-mediated IGF-1 gene transfer results in muscle hypertrophy and prevents aging-dependent loss in muscle mass (6). In another study on IGF-1 transgenic mice, revealed that IGF-1 overexpression first causes an increase in total DNA and RNA contents of the muscle and only several weeks after birth an increase in protein mass. IGF-1 gene was expounded as reflecting a primary effect on satellite-cell proliferation and fusion during the early postnatal stages, when satellite cells undergo active proliferation (7). Treatment of mouse C2C12 myoblasts with IGF-1 leads to increased proliferation on the cells and hypertrophy of resulting myotubes (8).

Satellite cells have been reported to play a crucial role in the hypertrophic response induced by viral-mediated gene transfer of IGF-1 in adult mouse muscles (9). Several studies have proven that adenovirus vectors encoding IGF-1 can induce growth muscle in animal models. However, adenoviral-mediated gene expression cannot be maintained for a very long period of time, and adenovirus vectors may induce an immune response in the host (8, 9, 10). In contrast, lentiviruses can integrate into the nucleus of the target cell and allow stable expression of transgene over the long term (11). Considering the key role of IGF-1 associated with growth, we hypothesized that overexpressing IGF-1 via a stable transgene expressing using a lentiviral vector may serve as a unique and promising strategy when *ex vitro* gene therapy is used for regeneration and hypertrophy of satellite cell. The aim of this study was cloning IGF-1 gene into lentiviral vector and production of recombinant virus and transfecting C2C12 myoblast cells with the recombinant virus. The present study was conducted to better understand the use of lentiviral vector to transfer IGF-1, the influence of recombinant lentiviral on IGF-1 expression in the C2C12 myoblast cells, and the effect of recombinant lentivirus on IGF1R mRNA and protein expression.

Materials and Methods

Cell Cultures

HEK293T (human embryonic kidney, ATCC CRL-3216) and C2C12 (mouse myoblast, ATCC CRL-1772) obtained from Mede Bioeconomy Company, Iran were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen, USA) and incubated at 37C° in a humidified 5% CO_2 atmosphere.

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Table 1. primer sequences for semi-qRT.

Target gene	Primer sequences	Amplification length (bp)
IGF1-F	AGGAGGCTGGAGATGTATTG	137
IGF1-R	TCGTGTTCTTGTTGGTAGATG	
IGF1R-F	TCCTGTGTTCTTCTATGTCC	141
IGF1R-R	GCTGTTATTTCTCTTTCTATGG	
β-actin-F	AGCCTCGCCTTTGCCGA	172
β-actin-R	CTGGTGCCTGGGGCG	

Construction of the pCDH-IGF-1-cGFP lentiviral vector

To amplify IGF-1 sequence, a pair of primers were designed and synthesized based on the IGF-1 cDNA sequences (NM-001111283.2). They both contains *Xba*I and *Not*I restriction enzyme site. The primers (forward primer , 5′- TCT AGA ATG GGA AAA ATC AGC-3′; reverse primer, 5′-GCG GCC GCT TAT TAT GTT TCC TGC ACT CCC TCT AC-3′) were used in a polymerase chain reaction (PCR) to amplify using pUC57 contains IGF-1 as a template using the following thermal cycling conditions : 95 °C pre-denaturation for 5 min; 95 °C denaturation for 30 s, 54 °C annealing for 30 s, 72 °C extension for 30 s for 30 cycles; 72 °C extension for 7 min. The PCR products were resolved with 1% agarose gel electrophoresis, and ~514 bp product was purified and cloned into the shuttle plasmid pCDH- cGFP(a gift from Tronolab) to construct the pCDH-IGF-1-cGFP lentiviral vector. In order to confirm the clone of containing a recombinant plasmid, enzymatic digestion was performed with *Xba*I and *Not*I enzymes and finally the recombinant pCDH- IGF-1- cGFP plasmid was Sanger sequenced.

Virus production

The lentiviral vector were transfected with three plasmids (pCDH- cGFP , psPAX2 and pMD2.G) in HEK-293T cells, using the standard calcium phosphate precipitation method according to Tronolab protocol with some modifications (12, 13). On day one, 5×10^6 HEK-293T cells were seeded in a 10 cm plate in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA). On the second day, 21 µg of transfer vector, 21 µg of psPAX2 vector and 15 µg of pMD2.G vector were mixed with transfection buffer and added drop wise to the cells. Transfection medium was replaced with fresh medium within 14 hours post transfection. The GFP expression was observed by fluorescent microscopy after 24h the packaged recombinant lentiviruses were harvested from the supernatant of cell cultures 48h post transfection bycentrifuged at 2000 rpm at 4°C for 5 min and filtering the supernatant through a 0.22 µm filter. The recombinant virus was stored at -70°C for subsequent experiments. Viral titer was determined with counting the number of GFP positive cells by flow cytometry.

Transduction of myoblast C2C12 cells

Myoblast C2C12 cells at a concentration of 1.5×10^5 were seeded in a 6-well plate. On the following day, the cells were transducted with recombinant lentivirus at a MOI of 20. After 24h, transduction media was replaced with fresh DMEM with 10% FBS The transducted cells were passaged every three days. The fluorescence properties of cGFP was examined under a LABOMED® Model T121100 fluorescent microscope (Labo, USA) and the taken images were evaluated by Image J software (14).

Chemiluminescence assay

To measure IGF-1 expressed protein concentration, supernatants from the myoblast C2C12 cells were collected 72 h post transduction and IGF-1 ELISA kit (ab100545) was used according to the manufacturer's instruction. The normal range of IGF-1 was 98-110 ng/ML for cells supernatant with this test.

Reverse transcription (RT-PCR) analysis

After 3 days of infection, total RNA was extracted from cells using RNeasy ® Plus Mini Kit (Alameda, United States). Complementary DNA (cDNA) was synthesized using QuantiTect Rev Transcription Kit (Qiagen, Germany), according to manufacture protocols. RT-PCR conditions were as follows: 1 cycle of 95°C for 5 min, and 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C 30 s and 1 cycle 72°C for 7 min. The primer sequences were used to RT-PCR are listed in Table 1. PCR products were run on 1.5% agarose gel and stained with DNA safe stain (Sinaclone, Iran). Gel images were visualized with a UV transilluminator (SABZ biomedical, Iran) and the integrated optical density (IOD) of each band was measured. The levels of IGF-1 and IGF1R mRNAs were normalized against β-actin.

Western blotting analysis

After 7 days of infection, the cells were lysed in 200 µl of RIPA buffer (Thermo Fisher Scientific, IL) supplemented with protease inhibitor. The cell lysates were centrifuged at 11000 rpm for 12 min at 4ºC and the supernatants were collected. Protein concentrations of the supernatants were determined with a BCA Protein Assay Kit (Thermo Fisher Scientific, IL). Protein lysates (30 µg/lane) were loaded onto 12% SDS–PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA). The membranes were blocked with 5% nonfat dried milk and immunoblotting was performed with IGF1R (Santa Cruz, CA) and β-actin (Santa Cruz, CA) antibodies at 1:1000 dilutions. Anti-rabbit IgG monoclonal antibody, conjugated with horseradish peroxidase at 1:2000 dilution. Finally the protein band was visualized by chemoluminescence reagent (ECL) and the integrated optical density (IOD) of each protein band was measured. IOD values were adjusted by internal standard β-actin.

Statistical analysis

All experiments were carried out in triplicate and all values were expressed as mean ± SD. Statistical diffe-

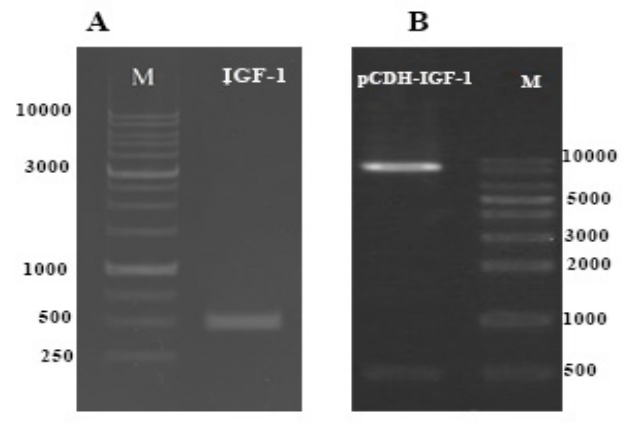

Figure 1. Construction of IGF-1 recombinant lentiviral vector. **A**: PCR product of IGF-1 and **B**: Recombinant pCDH-IGF-1 analyzed by restriction digest with *Xba* I and *Not* I, lane M: 1 kb DNA marker.

rences between groups were compared by one-way analysis of variance (ANOVA) using the ggplot package of R software. P<0.05 was considered statistically significant.

Results

Construction of IGF-1 recombinant lentiviral vector

A 514 bp amplicon was successfully cloned into the pCDH- cGFP vector (8190 bp) and the recombinant lentiviral vector was named pCDH-IGF-1- cGFP. Sequencing results confirmed that the cloned IGF-1 sequence was inserted correctly (data not shown). Digestion with *Xba* I and *Not* I enzymes also verified the accuracy of cloning **(**Fig. 1A-1B).

Packaging and titration of the recombinant lentiviral

The packaging of the recombinant lentivirus was verified using the expression of cGFP using the fluorescence microscope 24, 48 and 72 h after transfection, as shown in figure 2, more than 90% of HEK293T cells were transfected by the lentivirus pCDH- IGF-1. The lentivirus titer determination was based on the expression of cGFP in a cell-based assay. The titer of the recombinant virus was 10^6 IU/mL, whereas the empty vector pCDH had a titer over 10^7 IU/mL.

Recombinant lentiviral assessment based on cGFP expression

We initially investigated transduction efficiency of the recombinant lentiviral vector by analyzing cGFP expression in myoblast C2C12 cell. The number of cGFP-expressing cells and cGFP intensity was assessed 3 days post transduction. We noted a significant increase in the percentage of cells expressing cGFP through fluorescence intensity. Typically, at a MOI of 20, >80% of the transduced cells were found to be cGFP-positive using fluorescence-activated cell sorting analysis (fig. 3).

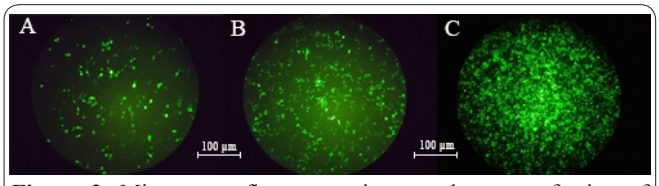

Figure 2. Microscopy fluorescent images show transfection of HEK-293T for achieving viral particles by pCDH- IGF-1. **A**: HEK- 293T at 24 hours after transfection, Panel **B**: HEK- 293T at 48 hours after transfection and **C**" HEK- 293T at 72 hours after transfection.

Recombinant lentivirus IGF-1 expression based on ELISA assay

To evaluate the effect of overexpression lentiviral vector on the level of IGF-1, we measured the concentration of IGF-1 produced in the transducted myoblast C2C12 supernatants, using commercially available ELISA kit. Our results confirmed that the IGF-1 level is increased significantly in the presence of recombinant lentiviral vector ($p < 0.01$) compared with the non-transducted group (mock) or pCDH-cGFP transducted group (negative control) (fig.4).

Recombinant lentiviral vector assessment based on RT-PCR analysis

Since the effect of IGF-1 on the muscle growth is mainly achieved through activation of the IGF1R gene, we investigated the mRNA expression of IGF-1 and IGF1R using RT-PCR. The expression levels of IGF-1 and IGF1R genes were normalized to the expression of the β-actin gene, as internal standard gene. Based on the results of gray scale scanning and comparative analysis with image J software, the expression of IGF-1 and IGF1R mRNAs significantly increased in cell transducted ith pCDH-IGF-1 recombinant construct rather than pCDH-cGFP (negative control) and non-transducted (mock) ($p < 0.01$) (Fig. 5) This result indicated that lentiviral pCDH- IGF-1 could overtranscribe the IGF-1 gene in myoblast C2C12 cells.

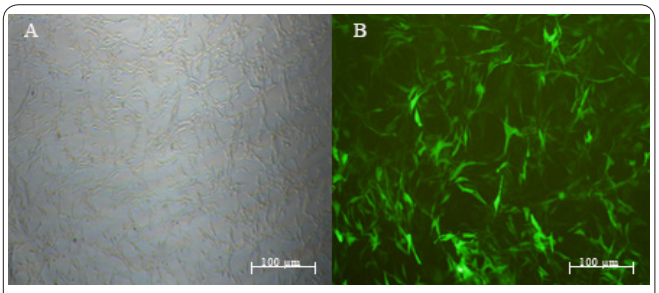

Figure 3. Transduction of myoblast C2C12 cells by lentiviral particles. A: C2C12 cells prior to transduction and B: transducted myoblast C2C12 cells by pCDH-IGF-1 lentiviral.

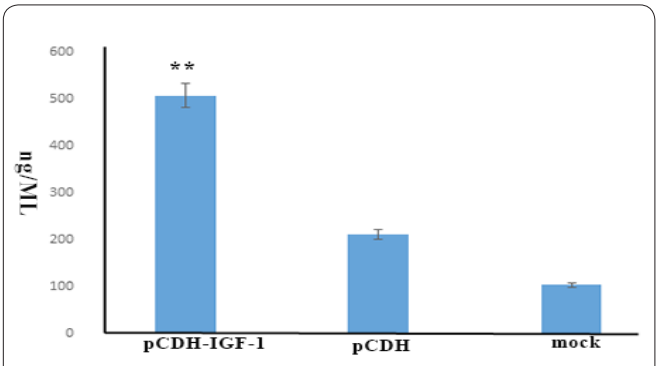

Figure 4. Effect of recombinant lentivirus on IGF-1 levels in Myoblast C2C12 cells. Bar graphs present levels of IGF-1 produced in experimental groups at 3 days following pCDH-IGF-1 transduction (Data are means ±SD, ** P<0.01).

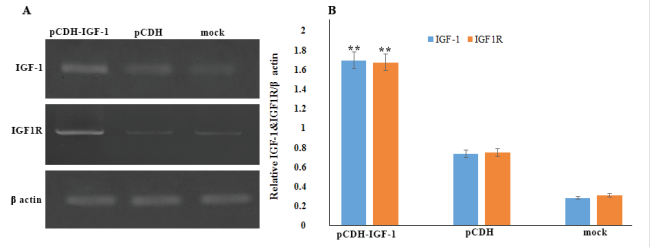

Figure 5. Effect of recombinant lentiviral vector on the mRNA expression of IGF-1and IGF1R in Myoblast C2C12 cells. **A**: RT-PCR detection of IGF-1and IGF-1R expression in Myoblast C2C12 cells after transduction**. B**) The IGF-1 and IGF1R mRNAs expression levels of different myoblast C2C12 cell groups were calculated using β-actin as an internal standard. (Data are means ±SD, ** P<0.01).

Recombinant lentiviral vector assessment based on Western blotting analysis

To elucidate the possibility that recombinant lentiviral vector lead to increasing the activity of IGF1R by overexpression of IGF-1, we quantified the IGF1R protein level by western blot assay in C2C12 cells that were transduced with recombinant lentivirus. Immunoblotting analysis demonstrated that the expression level of IGFR1 protein was markedly increased in the IGF-1 overexpression cells when compared with the pCDH-cGFP (negative control) and non-transducted groups (mock) (p>0.01). There was not statistical difference of IGF1R protein expression between the the pCDH-cGFP and mock groups (P>0.05), similar to results with RT-PCR analysis (Fig 6).

Discussion

The findings of this study have shown that by using lentiviral vector, mouse myoblast C2C12 cells can be successfully transduced and overexpress IGF-1 gene. based to the remarkable progress of biotechnology over the past decades, the ability to direct genetic changes at the molecular level has created a great revolution in biology (15). The idea that IGF-1 hormone/ protein could play an important role in promoting cell growth and division, began to develop in 1957 as the somatomedin hypothesis. Lentiviral vectors are essential tools for gene delivery because of their ability to integrate into host genomes with high efficiency. Recent studies have demonstrated high efficiencies for lentiviral transgenesis, including various animal species and cell line(16, 17). Based on the data published so far, IGF-1 has been suggested to play a role in the proliferation and differentiation of muscle satellite cells. Additionally, it has been demonstrated that the overexpression of IGF-1 can affect the proliferation and differentiation of muscle satellite cells and could be lead to hypertrophy in muscle. As part of this study, we show that lentiviral vector expressing IGF-1 mediated the overexpression of IGF-1 and IGF1R genes at mRNA and protein levels in the myoblast C2C12 cell line. IGF-1 is one of the most important growth factors in cellular processes of hypertrophy muscle. These processes can result from increased proliferation and differentiation in muscle satellite cells and these activations modulated by up regulation of IGF-1 gene (18). Meanwhile, IGF-1 has been noted as a predictor of muscle hypertrophy. In this study, we successfully transfect HEK293T cells with the recombinant vector and observed packaged viral particles with a high titer. Recombiant viral particles were able to infect myoblast C2C12 cells that were a suitable model cell type for the muscle cells. We assayed the effect of recombinant lentiviral vector on the expression of IGF1R and IGF-1 and found that recombinant lentiviral increased the expression of mRNA and protein of IGF1R. This gene is downstream of IGF-1 gene and the effect of IGF-1 on the regulation of gene expression is mainly achieved through activation of the IGF1R. This fact was corroborated with the finding of Rong et al where they used the recombinant retroviral vector mediated human expression in rat skeletal muscle (19). In other study, it was shown that overexpression of IGF-1 enhance the number of times that satellite cells can proliferate via PI3K signal transduction pathway (20). An effect of IGF-1 overexpression in transgenic mice muscle resulted in larger muscle fibers and enhanced muscle strength during aging (21). An association of increased serum IGF-1 concentration with overexpression of IGF-1 suggests that IGF-1 serum concentration is a useful indicator of average GH levels and the IGF-1 test is often used to evaluate growth factors efficiency. We showed that overexpression of IGF-1 markedly increased IGF-1 serum concentration in myoblast C2C12 cells. A similar result was observed on human skeletal muscle (22) .Altogether, results in our study and previous studies indicate that exogenous administration of IGF-1 stimulates gene expression of endogenous IGF-1 in muscle satellite cell and leading to muscle hypertrophy. To our knowledge, this is the first report of lentivirus-based vectors expressing growth factor. This method of transferring gene into muscle cells is more efficient and may be an effective strategy for delivering IGF-1 gene into muscle tissue of beef cattle to improve the accuracy of the beef cattle breeding process in the meat-producing strategies.

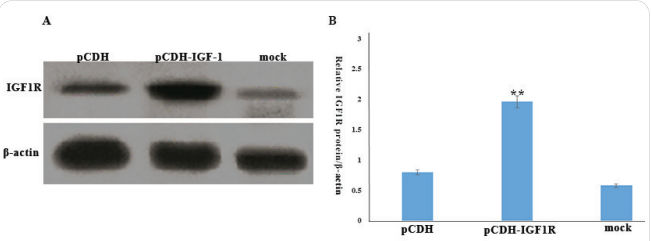

Figure 6. Effect of recombinant lentiviral on the protein expression of IGF1R in Myoblast C2C12 cells. **A:** Western blots analysis of different myoblast C2C12 cell groups. Equal amounts of cell extracts were used in all lanes. **B**: The IGFR-1 protein expression levels of different myoblast C2C12 cell groups were calculated using β-actin as a loading control. (Data are means ±SD, ** P<0.01).

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