

REVIEW

Myoblast Therapy: From Bench to Bedside

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Myoblasts are defined as stem cells containing skeletal muscle cell precursors. A decade of experimental work has revealed many properties of myoblasts, including the stability of resulting hybrid myofibers without immune suppression, the persistence of transgene expression, and the lack of tumorigenicity. Early phase clinical trials also showed that myoblast-based therapy is a promising approach for many intractable clinical conditions, including both muscle-related and non-muscle-related diseases. The potential application of myoblast therapy may be in the treatment of genetic muscle diseases, cardiomyocyte damaged heart diseases, and urinary incontinence. This review will provide an overview of myoblast biology, along with discussion of the potential application in clinical medicine. In addition, problems in current myoblast therapy and possible future improvements will be addressed.

Key words: Myoblast therapy; Duchenne muscular dystrophy; Myocardiopathy; Incontinence; Gene therapy

INTRODUCTION

Myoblasts are the quiescent skeletal muscle satellite cells. These cells, situated beneath the basal lamina that surrounds each myofiber, function for growth and repair of muscle (11,65). Within the last decade, many biological characteristics of myoblasts, along with the success of primary myoblast isolation, have been understood. Transplanted autologous myoblasts can survive and fuse with myofibers in vivo in syngeneic hosts; however, implanted cells were completely eliminated within 3 weeks in allogeneic hosts. Transplanted primary myoblasts, different from the transformed myoblast cell line, do not form tumors in immunodeficient mice. The properties of these cells after transplantation (the stability of resulting hybrid myofibers without immune suppression, the persistence of transgene expression, and the lack of tumorigenicity) inspired clinical researchers to explore the possibility of myoblast therapy for muscle diseases, based on the concept of many similarities between smooth muscle cells, cardiomyocytes, and myoblasts. Moreover, the approach of myoblast-based gene therapy may become an important application for additional non-muscle-related

diseases treated in the clinic (45). This review focuses on the recent development of myoblast therapy and discusses the substantial benefits of myoblast therapy in clinical medicine.

BIOLOGY OF MYOBLASTS

Human myoblasts are functional heterogeneity (11). Human self-renewing myoblasts have also been identified in the progeny of single human muscle satellite cells (HMSC) in culture (4). Native desmin-positive HMSC, cultured as single cells, yielded two major populations of myoblasts, alpha-sarcomeric (alpha-SR) actin-positive myoblasts and desmin-positive myoblasts. In appropriate culture conditions, alpha-SR-actin-positive myoblasts fused into myotubes, whereas a population of desmin-positive nonfusing myoblasts (NFMB) survived for weeks among the myotubes, without fusion. Upon isolation from myotubes, some of the NFMB resumed proliferation, resulting in fusing and nonfusing myoblasts, with the same cytoskeletal phenotypes as the progeny of native HMSC. Repetition of the self-renewal cycle yielded four cohorts of myoblasts, with an appearance of decrease in the yield of cells as the number of

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cycles increased. These results suggest that muscle stem cells are present among NFMB (4) and recent results have confirmed that human myoblasts contain muscle-derived stem cells (1).

In 1990, Dr. R. S. Walsh proposed that NCAM was a target cell surface antigen for the purification of muscle cells for myoblast transfer therapy (62). Since then, NCAM has been used for either identification of myoblasts or development of antibody-based purification. The isolation of human myoblasts requires three steps. In step 1, a single cell suspension of muscle tissue was mechanically dissociated and digested by collagenase and trypsin, and then cloned. In step 2, the developed clones were then tested for NCAM cell surface expression by use of cytofluorometric analysis with CD56 monoclonal antibodies. Finally, in step 3, only those clones with more than 98% strongly labeled positive cells were expanded for cell transfer therapy (6). In vitro propagated human myoblasts expressed the myoblast markers CD56 and desmin, but not TE7, a marker associated with the fibroblast phenotype (52).

Additional in vivo characteristics of human myoblasts have been evaluated in the RAG2(-/-)/gamma(-/-) and RAG2(-/-)/gamma(c)/C5 triple immunodeficient mouse models (12,13). Moreover, it is also known that myoblasts are immunogenic, and allomyoblast transplantation must be combined with immunosuppression (23). Therefore, physicians prefer autologous myoblast therapy for the treatment of muscle or nonmuscle diseases. The majority of the grafted cells quickly die, leaving only a minority responsible for new muscle formation. This minority is behaviorally distinct, with a slow divide in tissue culture, followed by a rapid proliferation after grafting. These in vivo findings verified that the subpopulation with stem cell-like characteristics is responsible for in vivo function (5).

MYOBLAST THERAPY FOR DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is a disease caused by the incapacity to synthesize dystrophin, which is implicated in the maintenance of the sarcolemma integrity. Experimental studies demonstrated that the dystrophin gene-transduced myoblast opened a new perspective in muscular dystrophy gene therapy (56,59). In a mouse model of congenital ptosis, a focal myopathy, the injected autologous myoblasts were found to be incorporated into muscle fibers within 2 weeks in the levator basal lamina, in contrast to the control. Electron microscopy of nearby sections showed these fibers to be maturing striated muscle, eluding to the fact that transplantation of normal myoblasts results in long-term restoration of dystrophin. Another intractable muscle disease, myotonic dystrophy (DM1), is caused by the

expansion of a trinucleotide repeat (CTG) located in the 3' untranslated region of the myotonic dystrophy protein kinase gene (DMPK). Gene-engineered myoblasts resulted in a preferential decrease in mutant DMPK transcripts, resulting in effective restoration of human DM1 myoblast functions, such as myoblast fusion and the uptake of glucose, which can ameliorate dystrophic muscle pathology at the cellular level (19).

In 1992, the first clinical trial in the US demonstrated the feasibility, safety, and efficacy of Phase II myoblast transfer therapy (MTT) (26). In this study, 5 billion normal myoblasts, from a male donor, were injected into each of 21 Duchenne muscular dystrophy (DMD) boys (aged 6–14 years), with or without histocompatibility. Results showed that MTT increased muscle strength in DMD: 81% of the muscles tested showed either an increase in strength or did not show continuous loss of strength. In addition, injections of 5 billion myoblasts have not provoked any immunological rejection symptoms in the Phase II subjects. The study also showed that donor cell rejection, by the recipient, in allogeneic myoblast therapy can be prevented with cyclosporine A, when properly managed. Moreover, first human myoblast transfer therapy efforts continue to show evidence of dystrophin after 6 years (25–27). These results suggest that autogenous myoblast transfer therapy is a useful treatment for congenital ptosis and other focal myopathies (3). And yet, in a pilot study, myoblast implantation did not improve strength of the implanted TA muscles. Another clinical study, conducted with monozygotic twin girls, both carriers of DMD, showed only a small increase in the number of dystrophin-positive fibers, in addition to the presence of numerous small type II fibers. Although the myoblast injections produced a significant force gain (12–31%) in wrist extension, there was no force gain for elbow flexion (36–38,57,58). The reason for this limited benefit has been known to be assessed to be inflammatory reaction, immunological problems, and low spread and poor survival of the injected myoblasts. Therefore, the myoblast-based therapy, when used for treatment of DMD, can still be optimized (50).

Several modified animal study protocols showed the improvement of these problems. For example, myoblasts genetically engineered to express an inhibitor of the inflammatory cytokine, IL-1, can improve the survival rate of the injected myoblasts (44). The induction of heat-shock proteins (HSPs) via heat-shock treatment was reported to improve the success of myoblast transplantation (8). Myostatin is known to be responsible for limiting skeletal muscle regeneration. Blocking the myostatin signal in mdx mice resulted in the following: increase of the size of the muscle fibers, increase in the fiber resistance to damage induced by exercise, and an improved success

of normal myoblast transplantation (7). Leukemia inhibitory factor (LIF) enhanced regeneration in injured and diseased muscle, followed by an increased number of dystrophic fibers expressing dystrophin, after myoblast transplantation (63). Coadministration of the anti-LFA-1 (TIB-213) mAb markedly reduced myoblast death at 3 days, without altering leukocyte tissue infiltration (20). Additionally, basic fibroblast growth factor, co-injected with primary myoblasts, improved significantly the level of expression of recombinant factor IX in vivo (64). Simultaneous administration of control-released hepatocyte growth factor (HGF) may also enhance the efficacy of skeletal myoblast transplantation through its antiapoptotic, angiogenic, and antifibrotic effects (53). CTLA4Ig coexpression, associated with a transient anti-CD154 treatment, can also prolong delivery of the recombinant protein, erythropoietin (Epo), via transfer of ex vivo modified cells in allogeneic recipients (22,47). These efforts may improve the clinical success of myoblast-based therapy for treatment of DMD in the future.

MYOBLAST THERAPY FOR MYOCARDIOPATHY

Chronic heart failure is the most common cause of hospitalization in the US, with about half a million patients each year. Cell-based cardiac repair shows the promise of rebuilding the injured heart from its component parts. Myoblast therapy for heart diseases is the most popular among the cell-based therapies (24). Numerous experimental successes for heart diseases have been demonstrated in rabbits (54,60), CHF147 Syrian hamsters (41), rat, and sheep models (2,9,30,39,42,43). Studies showed that the ACE inhibitor, in combination with myoblast therapy, significantly improved left ventricular function (42), which suggested that the cardioprotective effects of autologous SM cell transplantation appears more successful than those observed with ACE inhibitors. The improvement in ejection fraction is highly correlated with the number of injected myoblasts in the rat model. Therefore, the strategy, as used in the DMD model, to increase cell survival after transplantation may improve the clinical results. Evidence showed that coadministration of control-released hepatocyte growth factor, or hypoxia-inducible factor 1 α , enhanced the efficacy of skeletal myoblast transplantation, through its antiapoptotic, angiogenic, and antifibrotic effects in rats, with chronic myocardial infarction (2,53).

In 2001, Menasche's team in France first reported a success with implanted autologous skeletal myoblasts into the postinfarction scar, during coronary artery bypass grafting of remote myocardial areas, in a patient with severe ischemic heart failure. Five months later, there was evidence of contraction and viability in the grafted scar on echocardiography and positron emission

tomography (32,34). In another case, a 72-year-old man had autologous cultured myoblasts, from his vastus lateralis, injected into an area of transmural inferior myocardial infarction in nonreperfused scar tissue. Symptoms and left ventricular ejection fraction improved. When he died 17.5 months later, deemed unrelated to the cell transplantation after the procedure, the grafted postinfarction scar displayed well-developed skeletal myotubes, with a preserved contractile apparatus. Sixty-five percent of the myotubes expressed the slow myosin isoform and 33% coexpressed the slow and fast isoforms (44% and 0.6%, respectively, in skeletal muscle). The survival, along with a switch to slow-twitch fibers of the myoblast grafts, may be directly related to the sustained improvement in cardiac function (21). A further 10 patients were included, based on the following criteria: 1) severe left ventricular dysfunction (ejection fraction $\leq 35\%$); 2) the presence of a postinfarction akinetic and nonviable scar, as assessed by dobutamine echocardiography and 18-fluorodeoxyglucose positron emission tomography; and 3) an indication of coronary bypass in remote areas. Results obtained showed an average of 871×10^6 cells (86% of myoblasts) were obtained after a mean period of 16 days, and implanted, uneventfully, across the scar at the time of bypass. Although four patients showed delayed episodes of sustained ventricular tachycardia and were implanted with an internal defibrillator, the mean New York Heart Association functional class improved from 2.7 ± 0.2 preoperatively to 1.6 ± 0.1 postoperatively ($p < 0.0001$). In addition, the ejection fraction increased from $24 \pm 1\%$ to $32 \pm 1\%$ ($p < 0.02$) in all patients at an average follow-up of 10.9 months. Moreover, a blinded echocardiographic analysis showed that 63% of the cell-implanted scars (14 of 22) demonstrated improved systolic thickening (33,35).

Another clinical trial demonstrated that myoblast transplantation was feasible (48). In this clinical trial of 10 patients, one patient died of a recent infarction on day 7 postoperatively, due to a recent infarction located in a remote area of the left ventricle. The left ventricular ejection fraction increased from 25% to 40% (mean 35.2%) before the procedure to 29% to 47% (mean 42.0%) after the procedure, as noted during the 4-month follow-up visit ($p < 0.05$). This increased effect was maintained throughout the entire 12 months of follow-up. Sustained ventricular tachycardia was observed in two patients in the early postoperative period and in another two patients after 2 weeks of follow-up. This adverse event was controlled by amiodarone infusion.

A clinical trial in the US provided additional evidence for the feasibility and safety of autologous skeletal myoblast transplantation into infarcted myocardium (17). This Phase I nonrandomized, multicenter, pilot study enrolled 30 patients total, in which 24 of the pa-

tients were candidates for the elective CABG, and six of the patients were candidates for LVAD, as a bridge to heart transplantation. The patients had suffered myocardium infarction (MI) previously, and had significant reduction in their LV ejection fraction. The cultured myoblasts were injected into the epicardial surface of the infarction area, at the time of surgery. The preliminary results showed that the myoblasts were transplanted successfully, in all patients, without any acute injection-related complications, or significant long-term, unexpected adverse events. Histological evaluation in four of six patients who underwent heart transplantation provided documentation of survival and engraftment of the skeletal myoblasts, within the infarcted myocardium. Follow-up PET scans also showed new areas of viability within the infarct scar in CABG patients. Echocardiography was used to measure left ventricular ejection fraction (LVEF). In the 24 CABG patients, LVEF was improved from $28 \pm 9\%$ baseline to $35 \pm 8\%$ ($p = 0.02$) at a 1-year interval, and to $36 \pm 11\%$ ($P = 0.01$) at 2 years (16,17). However, the functional improvement was significant only in the concomitant CABG patients. It should be noted that in the absence of a control arm, the improvement in LVEF, LV dimensions, and LV volumes may be related only to benefits of the CABG. Therefore, regarding the efficacy, a meaningful conclusion cannot be drawn in this study. In order to show solid improvement of LVEF, further clinical trials should be considered in the absence of CABG and with a control group. Meanwhile, the potential adverse event, which was determined to be related to myoblast therapy, was nonsustained ventricular tachycardia. Fortunately, the adverse event of arrhythmia could be controlled by medication, or an ICD. Additionally, the author pointed out that the previous high incidence of arrhythmia was due to the small sample size. Nevertheless, the preparation of an antiarrhythmia therapy has been determined to be necessary before implementing myoblast transplantation.

Recently, a clinical study with five patients, using an invasive pressure-volume loop analysis, also demonstrated that the autologous myoblast transplantation improved pump function, with a tendency for improved systolic function, at up to 12 months into follow-up (51). Because all patients in this study had a MI at least 2 years before the cell transplantation, the myoblast therapy truly increased the contractility. They also demonstrated that the adverse event of arrhythmia did occur, but was found to be controlled by medication, without hospitalization or the implantation of a cardioverter-defibrillator. From the data of those pioneer clinical trials, there is an agreement that myoblast therapy will benefit those patients with reduced viable cardiomyocytes, and, at the very least, this approach will be an important option for those patients who are waiting for

heart transplantation. Based on these positive results, myoblast transfer is being researched in larger, randomized trials that use an average of 300 patients. The trials hope to thoroughly assess the efficacy of the procedure, in the clinical setting. The design of these studies must comply with the stringent methodological guidelines commonly used in drug trials, as this is the only means of accurately assessing whether to use the procedure, and to what extent, in order to impact the outcome of patients with advanced left ventricular ischemic dysfunction (31).

MYOBLASTS AS A TOOL FOR GENE DELIVERY

Myoblasts, the proliferative cell type of skeletal muscle tissues, become an integral part of the muscle into which they are injected. As such, they are potent tools for stable delivery of a gene of interest into the body, even in close proximity to circulation (18,19,40). Gene-engineered myoblasts afford a robust and long-lasting expression in muscle. The combined insertion of different gene constructs into single myoblasts, with their resulting progeny, allows the simultaneous expression of a functional and a reporter gene within the same cell in vivo. Additionally, the effects of timing and duration of gene expression can also be controlled by using tetracycline-inducible constructs and dosage effects, by selecting subpopulations consistently expressing distinct levels of growth factors. The modification of the vector system may also dramatically enhance transduction in differentiated myocytes in vitro and in skeletal muscle in vivo (18). Based on these biological advantages, myoblast-based gene therapy proves to be a promising approach to gene therapy.

A sustained systemic expression of factor IX was achieved by transplanting the genetically modified myoblasts into skeletal muscles in nude mice and SCID mice. This suggested that myoblast transplant may represent a useful approach to gene therapy for a variety of genetic diseases, including intrinsic muscle disease and defects in circulating proteins, as seen in the hemophilic (46,64). Recent attempts of gene-modified myoblast therapy have been expanded to insulin-dependent diabetes mellitus (IDDM) (49), neuropathy (29), anemia of end-stage renal failure (ESRF) with recombinant erythropoietin (EPO) (22,47), and human growth hormone (hGH) (14). This approach, which used transduced myoblasts, could also function as an alternative gene delivery system for therapeutic angiogenesis in patients, avoiding the toxicities seen with some viral vectors (61).

MYOBLAST-BASED THERAPY FOR URINARY INCONTINENCE AND OTHER DISEASES

Myoblast therapy for stress urinary incontinence is another popular topic, as it affects nearly 15 million peo-

ple (primarily women) around the world. Dr. Chancellor's team at the University of Pittsburgh demonstrated that the feasibility of myoblasts, the precursors of muscle fibers, injected periurethrally as a potential treatment of stress urinary incontinence, with results obtained from an animal study. Myoblast injection into the bladder wall improved detrusor contractility. The fusion of the injected myoblasts to form myotubes and myofibers in the smooth muscle layers of the lower urinary tract was seen in both the urethral and bladder walls. This suggests that myoblast injections can be used to enhance urethral closure and bladder function (10). Moreover, they recently confirmed the results of using human muscle-derived cells, pluripotent stem cells found in muscle, to cure stress urinary incontinence, by testing in animal models. This finding signals that these cells are prime candidates to treat this condition in adults (news report in September 18, 2005). Currently, clinical trials using muscle-derived cell therapy for incontinence have been initiated in Toronto, Canada.

On December 5, 2004, Dr. Frauscher at the University Hospital in Innsbruck, Austria, reported the first successful use of muscle myoblast stem cells for incontinence. The stem cells were removed from a patient's arm, cultured in a lab for 6 weeks, and then injected in the wall of the urethra and into the sphincter muscle. The result was increased muscle mass and contractility of the sphincter, along with a thicker urethra. Many patients showed no evidence of urinary leakage within 24 h of completion of the 15–20-min outpatient procedure. Twenty females, ages 36 to 84, who were experiencing minor to severe stress incontinence, participated in this research trial. One year after the procedure, 18 of the study's 20 patients remain continent. The research team has now planned to start using this technique at other centers, including three centers in Austria, two in Germany, one in Switzerland, and one in the Netherlands [The Annual Meeting of The Radiological Society of North America (RSNA)]. The expectation is to see more clinical trials being initiated throughout the world.

In 1997, Day reported the injection of a myoblast-based gene-delivery vehicle into a joint of both a newborn rabbit and an adult mouse with severe combined immunodeficiency. The results showed that myoblasts were transduced more efficiently than synovial cells, when using the same adenoviral preparation in vitro. After intra-articular injection, the engineered muscle cells adhered to several structures in the joint, including the ligament, capsule, and synovium. In addition, myoblasts fused to form many postmitotic myotubes and myofibers at different locations within the joint of the newborn rabbit, 5 days after injection. In the knee of the adult mouse, myoblasts fused and expressed the reporter gene for at least 35 days after injection. Stable myoblast-based gene delivery to the joint, which produces proteins, may be

an important therapeutic approach for musculoskeletal conditions with poor intrinsic healing capacity, in areas such as ligaments, meniscus, and cartilage (15). Researchers are continuing to improve current protocols for myoblast-based tissue engineering (28). Recently, myoblast-mediated gene therapy of nitric oxide synthase (NOS), an important transmitter that mediates smooth muscle relaxation and is essential for erection, injected into the corpus cavernosum of the penis, showed promise in correcting erectile dysfunction in adult male Sprague-Dawley rats (55).

In conclusion, myoblasts are adult stem cells and include skeletal muscle cell precursors. Although myoblasts are committed myogenic cells, these cells can be induced to differentiate, and can stably survive in the form of hybrid myofibers. After fusion, hybrid myofibers maintain their muscle character and in vivo contractility. Because we can isolate, purify, and expand human myoblasts in vitro, myoblast therapy has been brought to early phase clinical trials for several indications, and has been shown to be a positive trend for many intractable clinical conditions, including muscle- and non-muscle-related diseases. The popular clinical trials of myoblast therapy are in the treatment of genetic muscle diseases, cardiomyocyte-damaged heart diseases, and urinary incontinence. However, in order to gain a more definitive test of the efficacy of myoblast therapy in the setting of chronic ischemic heart diseases, the design of clinical trials should include a protocol with randomized groups and without CABG. The protocol of further clinical trials should also include a procedure to minimize the adverse event of ventricular tachycardia, though all of the reports showed safety of myoblast therapy so far. Moreover, to improve the clinical effects, problems such as inflammatory reactions, immunologic rejection, low spread and poor survival of the injected myoblasts, and synergic contractility with host cardiomyocytes need in vitro and in vivo solutions. The mechanism of myoblast therapy for heart damage needs also to be investigated. Because the application of myoblast therapy is substantial, more fundamental research regarding those problems and clinical trials is warranted.

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