Surg Today (2010) 40:902–908
DOI 10.1007/s00595-009-4242-z

SURGERY TODAY

Review Article

Myoblast Transplantation: A Possible Surgical Treatment for a Severe Pediatric Disease

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Abstract
Duchenne muscular dystrophy (DMD) is a genetic X-linked recessive orphan disease that affects approximately 1 in 3500 male births. Boys with DMD have progressive and predictable muscle destruction due to the absence of dystrophin, a protein present under the muscle fiber membrane. This absence induces contraction-related membrane damage and activation of inflammatory necrosis and fibrosis, leading to cardiac/diaphragmatic failure and death. The authors support the therapeutic role of myoblast transplantation in DMD, and describe the history and rationale for such an approach.

Key words Myoblast · Duchenne muscular dystrophy · Exon skipping · Tacrolimus · Cyclosporine

Organ Versus Cell Transplantation

Attempts are currently underway to expand the surgical experience from solid organ transplantations to stem cell replacement or repair of tissues or parenchyma whose function has been impaired. This would be done without removing them en bloc as in the classic liver, heart, and kidney standard transplantation procedures, but just by grafting new cells while leaving the histological environment, namely the stroma, nerve, and vascular supply, unchanged. In contrast to the solid organ transplant philosophy, this more recent cell-seeding strategy requires specific timing to be effective because if the degenerative process has progressed too far, then the chances of a functional success are greatly reduced.

One of the most intriguing areas of potential progenitor cell transplant is the striated voluntary muscular tissue affected by genetic diseases, where the clinical feasibility has been reviewed based on 20 years of experimental background. However, no definite transplant protocol has yet been established owing to delays in performing phase 1/2 clinical trials.

The aim of this article is therefore to stress the opportunity offered by MT to restore function of genetically damaged muscles and to urge dedicated teams of surgeons and biologists to perform clinical trials to demonstrate the long-term benefits of this procedure, and to definitively reverse the muscle damage without having to repeat the allogeneic transplantation.

Reprint requests to: B. Palmieri
Received: September 17, 2008 / Accepted: August 26, 2009

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy is a fatal hereditary disease for which there is currently no treatment. Progressive muscle weakness starts at age 5 years, thus leading to wheelchair confinement by age 10–12, and death from respiratory complications or secondary heart disease between the age of 17 and 30 years. Duchenne muscular dystrophy is caused by an X-linked genetic defect leading to the absence of dystrophin (Dys).[1] The absence of dystrophin under the plasma membrane makes the muscle fibers more vulnerable during contraction.[2] Damage to muscle fibers is present as soon as the child is born, as demonstrated by the high creatinine kinase (CK) level in serum following its release from damaged muscle fibers. Damaged fibers are repaired by proliferation and fusion of peripheral stem-cell-like cells, called "satellite cells." These satellite cells are called myoblasts when they proliferate. Unfortunately, in the case of DMD the repaired muscle fibers still lacks dystrophin and thus remains vulnerable during subsequent contractions. This leads to repeated cycles of damage and repair. At each of these cycles, the satellite cells have to proliferate to generate myoblasts for repair. These frequent cycles of proliferation lead to

their senescence.[3] This results in progressive loss of regenerative potential by age 4 to 5 years, as indicated by the progressive difficulty with aging to proliferate myoblasts from muscle biopsies of DMD patients.[4] Therefore, damaged muscle fibers are no longer adequately repaired and thereafter become progressively replaced by fat and conjunctive tissue.

Myoblast Transplantation

Various types of cells are being investigated for cell therapy of muscular dystrophy. This subject has been recently reviewed.[5] Among these cells are freshly isolated satellite cells; myoblasts proliferated in vitro from these satellite cells, mesoangioblasts, pericytes, and pluripotent cells called muscle-derived stem cells (MDSCs). Cellular therapy takes advantage of the fact that these various types of cells derived from a healthy donor carry the normal dystrophin gene can fuse with damaged DMD muscle fibers. This fusion introduces the normal Dys into the existing muscle fibers and protects them from further damage. Indeed, transplantation of wild-type syngeneic myoblasts was shown as early as 1989 to restore Dys expression in immunodeficient mdx mice, a model of DMD.[6] Good transplantation results were also obtained by different research groups in mdx mice immunosuppressed with cyclosporine or tacrolimus.[6–9]

The success of myoblast transplantation (MT) in mice led to the investigation of MT in monkeys, where the immune system and the size of the biceps muscle are comparable to humans. Tacrolimus treatment also prevents the rejection of MT.[10–13] Up to 75% of the fibers throughout the entire biceps of monkeys are of hybrid origin 1 month after MT.[14] These hybrid fibers are present over one year after MT. This clearly demonstrates that transplanted myoblasts can fuse efficiently with normal muscle fibers in primates.

Early clinical trials of myoblast transplantation in Duchenne and Becker muscular dystrophies (between 1990 and 1998) were all done on patients younger than 18 years, and unfortunately produced only very limited positive results.[15–22] During that period, Karpati et al.[18] transplanted 55 million allogeneic normal myoblasts throughout the biceps of eight young DMD patients immunosuppressed with cyclophosphamide over 6 or 12 months. They did not detect any increased expression of dystrophin in the muscle biopsies of the patients. Two years later, this research group demonstrated that the absence of success in this clinical trial was due to the fact that cyclophosphamide killed all the proliferating cells, including the myoblasts.[23] Gussoni et al.[15] transplanted 100 million myoblasts to eight DMD patients aged 6–10 years and administered cyclosporine for immunosuppression. They reported the expression of the normal dystrophin transcript in the muscle biopsies obtained 1 month after the transplantation in three patients. Gussoni et al.[24] re-examined the muscle biopsies from six of their DMD patients a few years later, using a fluorescence in situ hybridization (FISH)-based method. Donor nuclei were detected in all biopsies analyzed, including nine where no donor myoblasts were previously thought to be present. Three patients showed that more than 10% of the original number of donor cells was present 6 months after implantation. Half of the detected donor nuclei were fused into host myofibers, and of these nearly 50% produced dystrophin. These findings demonstrate that although donor myoblasts have persisted after injection, their microenvironment influences whether they fuse and express dystrophin. The Mendell group injected 110 million donor myoblasts once a month for 6 months (total 660 million myoblasts) to the biceps brachii muscles of one arm of 12 DMD boys aged between 5 and 10 years immunosuppressed throughout this period with cyclosporine. For each transplantation session the myoblasts were injected at 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns. They observed 10% dystrophin-positive fibers in 1 out of 12 patients.[17] They were, however, able to demonstrate that the dystrophin was of donor origin because of the presence of a dystrophin epitope coded by an exon deleted in the patient genome. A few years later, Hong et al.[25] demonstrated in mice that cyclosporine blocked differentiation and induced myoblast apoptosis at an early stage of muscle differentiation. This probably accounts for the poor results obtained in the clinical trials that used this immunosuppressive drug. This group transplanted myoblasts obtained from perfectly human leukocyte antigen-compatible donors, assuming that immunosuppressive treatment would not be required. Some dystrophin-positive fibers were observed in a few patients.[19] However, it was not possible to demonstrate that the fibers were of donor origin because patients with an identified deletion were not selected. This first clinical trial also detected the presence of antibodies against dystrophin. Subsequent studies demonstrated that the transplantation of major histocompatibility complex-compatible myoblasts without immunosuppression led to a rejection due to the minor antigens in mice.[26] Further experiments in animal models permitted the identification of two additional problems of these early clinical trials. The first problem was that myoblasts do not migrate away from the injection trajectories, thus a high density of injection is required to obtain a high percentage of hybrid fibers.[12,14,27] The second problem is that a high percentage of the transplanted cells died during the first few days following transplantation.[28–31] Therefore, 30 million myoblasts had to be injected per cm^3 of muscle to obtain good transplantation results in monkeys. These

early MT clinical trials produced at best very limited results because of inadequate immunosuppression, an insufficient number of transplanted cells, and insufficient distribution of the cells.[15,17,19,20,32]

A more recent clinical trial transplanted myoblasts into only 1 cm^3 of muscle with a follow-up tacrolimus immunosuppression of only 1 month.[33] This clinical trial demonstrated that the transplantation of normal allogeneic myoblasts restores the expression of dystrophin in up to 26% of the muscle fibers within 1 cm^3 of the injection site. A concurrent compassionate treatment in a 26-year-old patient restored the expression of dystrophin in 34.5% of the muscle fibers.[34] This observation was made in the 1 cm^3 of muscle that was biopsied; however, this muscle was transplanted with myoblasts throughout its volume and thus the muscle biopsy probably reflects the results that would be obtained throughout that muscle. Some small-diameter muscle fibers that are apparently completely of donor origin were observed in a recent clinical trial.[33] Importantly, some of the transplanted myoblasts did not fuse immediately and remained as quiescent satellite cells, which will be able to repair subsequent damage induced by normal muscle activity.[35,36] The Morgan group recently demonstrated that myoblasts transplanted in an mdx mouse muscle can be expanded following a muscle biopsy and then retransplanted with success, suggesting that they function as satellite cells.[37] Recent results confirmed that the donor cells are not only in a satellite cell position in mice but are Pax-7 positive and MyoD negative, confirming that they are satellite cells (unpublished results). Moreover, donor cells were also observed in a satellite cell positions in a muscle biopsy of the patients who participated in a 2005 clinical trial (unpublished results).

Other Treatment Options for DMD

Several approaches are currently being pursued for the treatment of DMD. Gene therapy aims to introduce the *Dys* gene directly into the patient's muscle fibers. However, as in other forms of gene therapy, prolonged gene expression, avoiding a host immune response to the vector and widespread distribution, remains problematic. Indeed the most likely viral vector to treat DMD is currently the adeno-associated virus (AAV); however, Chamberlain's group recently reported an immune response again the AAV vector in the dog and suggested that immunosuppression may be required in an eventual clinical trial.[38] Moreover, the AAV vector can contain only a truncated version of the dystrophin gene (called micro-dystrophin). This newly expressed protein differs from the truncated endogenous versions of dystrophin present in a few fibers of the patients. These so-called revertant fibers are caused by somatic mutations leading to the skipping of one or several exons. It is possible that new junction peptides may provoke an immunological reaction to this "neoantigen" since the micro-dystrophin differs from the patient revertant dystrophin. A new approach to gene therapy, based on exon skipping, bypasses mutant stop codons and allows for the expression of truncated Dys, which is likely to result in milder disease phenotypes (such as those seen in Becker forms of dystrophy).[39–41] Moreover, since the Dys expressed may be identical to that endogenously expressed by rare revertant fibers, immunogenicity might be avoided but the development of tolerance by the presence of revertant fibers has not as yet been proven. However, uncertainty remains about the long-term potential toxicity of the morpholino oligonucleotides used to induce the exon skipping, as they are not degradable by the cells and may thus eventually accumulate in the nuclei.[42–44] Moreover, exon skipping is not a solution for all DMD patients and for several other recessive dystrophies.

Although mesoangioblasts (blood vessel-derived cells) may be delivered systemically to restore the expression of dystrophin in dystrophic dogs,[45] the intramuscular delivery of myoblasts has proven effective and safe in DMD patients and should not be given up until another treatment has been proven more effective in patients. Direct intramuscular myoblast transplantation may be useful to increase the strength of specific muscles in DMD or in other muscular dystrophies with more localized muscle weakness.

Can MT Improve the Disease Phenotype?

There is evidence supporting the beneficial effects of MT. Damage is observed only in Dys-negative fibers but not in the Dys-positive fibers resulting from MT when mdx mice are subjected to eccentric exercise 1 month following MT.[46] Therefore, MT protects the muscle tissue of mdx mice from the mechanical stress that serves as the trigger for myofiber necrosis in DMD. Moreover, some of the grafted myoblasts have also been shown to form new muscle fibers in a mouse model.[37]

Discussion

New Clinical Trial of MT?

This group is currently trying to conduct a second clinical trial to verify whether MT can increase the strength of one DMD muscle, but there are limitations imposed by the human ethics committee and the regulatory agency. The current postulated clinical trial aims to transplant myoblasts in only a single small muscle (the

extensor carpi radialis), while employing a 6-month tacrolimus immunosuppression protocol to evaluate whether this procedure can improve the strength of that muscle. However, the local ethics committee currently restricts this clinical trial to only patients older than 18 years. Moreover, the regulatory agency also requires that the DMD patients older than 18 years are not on a respirator and are not seropositive for the cytomegalovirus (CMV). While these restrictions are justified because of the increased risk of infection and the risk of reactivation of the CMV, this greatly limits the number of patients that can participate in a clinical trial, since most DMD patients older than 18 are on respirators and 70% of adults are CMV seropositive.

Duchenne muscular dystrophy patients older than 18 years are in a very advanced stage of the disease with their muscle being largely infiltrated by fat and conjunctive tissue, making the demonstration of an improvement of strength extremely difficult. If the function is preserved or improved during the follow-up, it would be wise to transplant myoblasts into other muscles of the same patient to further prevent muscle failure while maintaining adequate immunosuppressive treatment. However, the current ethics and regulatory approval does not include this possibility. The risks associated with immunosuppression should therefore be justified given the extremely low quality of life of DMD patients.

One of the main conceptual objections to MT clinical trials is the concern about side effects of immunosuppressive therapy, for a treatment that has not yet been proven as effective to increase the strength of the patients. Indeed the average life expectancy of DMD with new technological respiratory support and dedicated medical care is now over 25 years. Therefore, even if the quality of life is progressively deteriorating due to the impairment diaphragm and heart muscles, human ethics committees are reluctant to permit even a 6-month period of immunosuppression with tacrolimus because of the potential adverse effects associated with this drug (increased risk of cancer, diabetes, neurotoxicity, etc.). The ethics committees are also reluctant to permit the inclusion of young DMD patients because, except for need to use a wheelchair from about 11 years of age, these patients do not usually require specific medical assistance.

The philosophy and ethics of solid organ transplant is that only a life-threatening organ failure justifies the risks of transplant challenge. One exception is kidney insufficiency, where dialysis and artificial kidney support provide the choice to avoid transplantation but at the price of a very poor life quality, a very intensive monitoring and treatment schedule, and a high complications rate. A similar exception should be made for DMD patients. Indeed although their life can be extended to around 25 years with respiratory assistance, their quality of life past 17 years old is progressively deteriorating as they become totally unable to move and eat by themselves, and eventually even unable to control their own electric wheelchair. Their parents have to feed them, change their diapers, and turn them in their bed several times each night. Although MT does not currently aim to prevent death, it may permit patients to improve their strength in several muscles, thereby enabling them to achieve a greater autonomy that greatly justifies the risks associated with immunosuppression.

Tacrolimus Immunosuppression in Children

Numerous studies over the last 10 years have accumulated significant experience with tacrolimus side effects. The list of centers where tacrolimus is being used for transplantation in children is already quite long. The main adverse effects are increased Epstein–Barr virus (EBV) and CMV infections.[47] One of the clinical trials showed 13.7% lymphoproliferative diseases, but 81.3% of these were controlled by drug treatment.[48] A comparison between tacrolimus and cyclosporine in 70 children aged 6.5 years undergoing heart transplant showed a comparable nephrotoxicity between the two groups 6 years after transplantation.[49] Another investigation in pediatric liver transplants switched 42 patients from cyclosporine to tacrolimus. A 16-month follow-up showed that the main side effects were arterial hypertension (9.2%), liver toxicity (2.3%), and cosmetic damage (4.8%).[50] The hypertrophic cardiomyopathy associated with tacrolimus use after three small bowel and two liver transplants in five children aged 23 months with a follow-up of 11 months was detected and treated by reducing or stopping tacrolimus administration.[51] One hundred and three pediatric kidney transplant patients younger than 80 months were followed up for 60 months during treatment with oral tacrolimus. Only three patients developed lymphoproliferative disease due to EBV infection. The incidence of type II diabetes was lower in children with tacrolimus than in those with cyclosporine. Tacrolimus was superior to cyclosporine in preventing acute kidney rejection when used in combination with azathioprine and corticosteroids, with a better graft survival at 4 years.[52] An investigation of 49 patients aged 6.1 months with heart transplant with a follow-up between 3 and 96 months showed that the most frequent side effects were anemia, renal toxicity, hyperkalemia, and gastrointestinal and allergic problems. Late deaths (1.4%) were mainly caused by severe infection, coronary obstruction, lymphoproliferative disease, and mitochondrial myopathy.[53]

This overview demonstrates that the use of tacrolimus in infants and children shows an acceptable rate of complications, considering its advantage in rejection control effectiveness in the major organ transplant area.

Considering that DMD patients are otherwise healthy, tolerance to tacrolimus in this cohort should thus be even better. Furthermore, in the near future the immunosuppressive therapy used to prevent myoblast rejection in DMD patients might be modified to a multitherapy approach with lower doses of each compound, excluding steroids, which in the current protocols are responsible for some relevant untoward effects. The availability of new immunosuppressive compounds such as sirolimus and everolimus, with specific antitumor capacity, should be also evaluated to further reduce the oncological risk. It may also be possible to develop a sustained immunological tolerance toward the donor myoblasts and the hybrid muscle fibers that they form, since this goal has been achieved in mdx mice.[54–56]

Importantly, the trials with type I diabetes involved children with a disease that can be controlled (although imperfectly) with insulin administration, and the progression of this disease is certainly much less severe than DMD. Indeed tacrolimus is currently being used in children for the treatment of autoimmune diseases such as myasthenia gravis,[39] nephritis due to systemic lupus erythematosus,[40] focal segmental glomerulosclerosis,[41] and chronic urticaria resistant to other treatments,[42] which are much less severe than DMD. The incidence of lymphoma in pediatric renal transplantation was of 0.3% at 1 year in recent immunosuppressive protocols containing tacrolimus.[43] Importantly, all available evidence suggests that it is cumulative immunosuppression and not a specific drug that promotes the development of lymphoma.[44,45] In this regard, renal transplant recipients, including children, receive a combination of immunosuppressive drugs that may include T-cell depleting monoclonal antibodies that increase the risk of lymphoma. The incidence of lymphoma appears to be much lower in trials using calcineurin inhibitors as single agents in children. For example, 20 children (averaging 11 years of age) with focal segmental glomerulosclerosis received tacrolimus plus corticosteroids for 12 months and none developed lymphoma.[41] Therefore, the incidence of lymphoma in DMD patients being treated with tacrolimus as a sole immunosuppressive agent should exhibit a low incidence of malignancy. The fact that heart and lung transplant recipients develop more lymphomas than those who receive kidney grafts is in fact attributed to the less intensive immunosuppression used in the latter case.[47]

Conclusion

This is now the beginning of the stem cell era where, especially in central nervous system diseases, the goal to regenerate a neural network is strongly advocated while in the field of heart pathology, myocardial regeneration after acute or chronic failure or infarction is actively being attempted. In the case of skeletal muscle diseases, the concept of repairing defective muscle fibers is possible by introducing through the skin, possibly with a robotic arm, a great number of myoblasts in close proximity (1 mm apart). In the future, MT should be administered as soon as the first symptoms of muscular failure appear, for two reasons: (1) a lower number of cells will be required due to underdeveloped muscular mass, and (2) a better quality of viable muscular tissue will be colonized by the donor myoblasts.

There is evidence that some of the transplanted myoblasts form satellite cells can repair damaged muscle fibers, thus suggesting that the retransplantation of the same muscles will not be necessary, and such MT benefits will therefore last a lifetime. It is necessary to demonstrate the improvement of the contractile function and motility improvement in the younger patients. Only such an investigation will definitely confirm the feasibility of MT. Current efforts will continue to make MT an easy, safe, and effective standard procedure.

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