

Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy

Palmieri B, Tremblay JP, Lodi D. Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy. *Pediatr Transplantation* 2010. © 2010 John Wiley & Sons A/S.

Abstract: DMD is a genetic X-linked recessive disease that affects approximately one in 3500 male births. Boys with DMD have progressive and predictable muscle destruction because of the absence of Dys, a protein present under the muscle fiber membrane. Dys deficiency induces contraction-related membrane damages, activation of inflammatory-necrosis-fibrosis up to the cardiac-diaphragmatic failure and death. This review supports the therapeutic role of MT associated with immunosuppression in DMD patients, describing the history and the rationale of such approach. The authors underline the importance to evaluate a protocol of myoblast intradermal multi-injection to apply in young DMD patients.

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Key words: myoblasts – Duchenne muscular dystrophy – exon skipping – tacrolimus – cyclosporine – transplantation

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Accepted for publication 25 June 2010

DMD

DMD is a severe recessive X-linked muscular dystrophy characterized by progressive muscle degeneration. High serum levels of CK, a marker of muscle injury, in new borns demonstrate that muscle fiber damages begin precociously. Progressive muscle weakness starts at five yr and leads to wheelchair confinement at 10–12 yr. Patients die from respiratory complications or secondary heart diseases between 17 and 30 yr. DMD is caused by genetic defects on *dmd* gene that codifies for Dys (1). Dys forms part of a large, tightly associated and oligomeric complex of proteins, the DGC, located under the plasma membrane (2).

Abbreviations: AAV, adeno-associated virus; CK, creatinine kinase; CMV, cytomegalovirus; Cy, cyclosporine; DGC, dystrophin–glycoprotein complex; DMD, Duchenne muscular dystrophy, Dys, dystrophin; GvHD, graft-versus-host-disease; MDSC, muscle-derived stem cells; MHC, major histocompatibility complex; MT, myoblast transplantation; SC, stem cell; SCT, stem cell transplantation; SOT, solid organ transplantation.

Being a structural component of myocytes, mutations of Dys cause cellular damages and, macroscopically, fragility and vulnerability of muscle fibres during contraction (3). Fibers damaged are physiologically repaired by proliferation and fusion with satellite cells, peripheral stem cell-like. When proliferating, satellite cells are called myoblasts (4, 5). Unfortunately, in DMD patients, the repaired muscle fibers still lack Dys and thus remain vulnerable during subsequent contractions. This triggers continuous damage–repair cycles and rapidly leads myoblasts to senescence (6). Muscles loss regenerative potential by age 4–5 yr, as indicated by the progressive difficulty with aging to proliferate myoblasts from muscle biopsies of DMD patients (7). Thus, damaged muscle fibers are no longer adequately repaired and are progressively replaced by fat and connective tissue.

Prior experimental approaches

Cellular therapy was the first approach adopted to treat DMD. Cellular therapy takes

the advantage to protect muscle fibers from degeneration by fusion with healthy cells. Indeed, transplantation of wild-type syngeneic myoblasts was shown as early as 1989 to restore Dys expression in immunodeficient mdx mice, an animal model of DMD (8). Good transplantation results were also obtained in mdx mice immunosuppressed with Cy or tacrolimus (8–11).

Acsadi et al. (12) injected Dys with a plasmid vector by intramuscular injection, but only 1% of myoblasts were able to express the protein and this was not sufficient to obtain a clinical effect.

However, early clinical trials of MT (from 1990–1995) produced limited positive results. During that period, our group transplanted myoblasts obtained from a perfectly MHC compatible donor and we observed some Dys-positive fibers in a few patients (13). However, we were not able to demonstrate that they were from donor origin. Mendell et al. (14) combining Cy immunosuppression with MT observed 10% donor Dys-positive fibers in one of 12 patients. Dys was certainly of donor origin because sequencing analysis showed the presence of an exon deleted in the patient genome. Unfortunately, these early MT clinical trials produced poor results because of inadequate immunosuppression, insufficient number of transplanted cells and insufficient distribution of the cells (13, 15–18).

In spite of high cell death rate during graft, several of the transplanted myoblasts in mice remained quiescent cells, which will be able to repair subsequent damage induced by normal muscle activity (19, 20).

Kinoshita et al. (21) perfectly controlled the immune response to MT in monkeys by tacrolimus.

Several studies were performed on animal models to investigate the efficacy of partial or whole muscle transplant. These works were based on the ablation of whole myobundles from normal animal and the transplantation of slices in mdx mouse. Immunocompromised mdx mice grafted with muscle slices showed huge implanted myofiber degeneration associated with new muscle presence within 10 days, predominantly from donor cells. From muscle slices, cells emigrated and fused with host counterparts. No long-term immunological rejection of the donor myoblasts was there (22).

On animal DMD models, muscle slice graft seemed to be necessary for tissue repopulation. Apparently, myoblasts' isolation and purification rendered them largely incapable of proliferation and fusion in recipient environment, while elsewhere degeneration and regeneration were occur-

ring. Myoblasts were certainly capable of forming myotubes *in vitro*. Perhaps, the isolation procedure produced subtle changes in the myoblasts that rendered them either more visible to the host immune system or less viable for fusion *in vivo*. Myoblasts survival improved significantly if injected in immunocompromised host (23).

Likely, muscle slice graft could not enter in Duchenne therapy. Indeed, the transplantation of donor muscle strips would be limited to the larger and the surgically more accessible dystrophic muscles. Another obstacle is represented by compatible-muscle slice availability. Obtaining a large amount of muscle strips by donor is difficult, and biotechnologies are not able to reproduce myofibers in laboratory yet. Ultimately, there are not clear surgery conditions such as slice dimension and placement, pre- and post-graft immunomodulation protocols, to permit a sufficient long-term graft vs. host fusion (22).

In 2001, Smythe et al. discovered that MT results could be improved by MyoD decrease. In fact, migration into host muscle was enhanced without MyoD. So, modulation or administration of mitogens could become an important support to MT. However, it is necessary to pay attention that cells' proliferation and/or cells' fusion delayed in enhancing migration into host muscle *in vivo* are based on opposite pathways (24).

The success of MT in mice led our group to investigate MT in monkeys, where the immune system and the size of the biceps muscle were comparable to humans. Up to 75% of the fibers throughout the entire biceps of monkeys were of hybrid origin one month after MT (25). Hybrid fibers were present for over one yr after MT. This clearly demonstrated that transplanted myoblasts can fuse efficiently with normal muscle fibers in primates (26, 27).

Hong et al. (28) demonstrated that the success of MT depended on the immunosuppressive drug used. Indeed, several, such as cyclophosphamide, killed proliferating myoblasts and other such as Cy led to myoblasts apoptosis during their terminal differentiation.

Likely for the unsatisfactory results obtained by cell therapy, from 2003 to 2006, other approaches took place to treat DMD. Gene therapy aimed to introduce the Dys gene directly into the patient's muscle fibers. However, long gene expression triggered a host immune response against the vector. The first vector used in DMD gene therapy was plasmid. On animal models, Dys expression increased to 10% in the muscle fibers after intramuscular or intravenous/arterial injection of plasmid (12, 29), and it was

detectable for at least six months (30). A phase I clinical trial showed a low level of Dys expression for up to three wk in radial muscle fibers injected by plasmid-mediated delivery Dys cDNA. No adverse effects were noted, but also no clinical significant improvements were observed (31). Indeed, the most hopeful viral vector to treat DMD is currently the AAV; however, Chamberlain et al. recently reported an immune response against the AAV vector in the dog. Immunosuppression will be necessary in an eventual clinical trial (32). Moreover, the AAV vector could contain only a truncated version of the Dys gene (called micro-Dys), being wild-type protein excessively long. The newly expressed protein differed from the truncated endogenous Dys, called revertant. Revertant Dys lacked of one or several exon. Being the micro-Dys differs from the patient revertant Dys, it was possible that new junction peptides could cause an immunological reaction to this “neoantigen” (33–35).

Pharmacological approaches were variable. Many compounds such as vitamin E, selenium, myostatin, glucocorticoids, antibiotics and up-regulator of Dys-related protein were investigated, but no one improved stably the condition of DMD patients or decelerated disease progression (36).

Systemic injection of mesoangioblasts (blood vessel-derived cells) restored the expression of Dys in dystrophic dogs (37).

Current approaches

Gene therapy returned actual in 2007 with the exon skipping, a technique that permit to substitute a genetic sequence and bypass mutation or stop codon. The rationale is to produce a protein, full length or not, that could reduce the severity of pathology such as in Becker dystrophy. No evidence of immunoreactivity against corrected myoblasts was observed; rather, the development of tolerance of the revertant fibers has not been proven yet. However, long-term potential toxicity of the morpholino, oligonucleotides used to induce the exon skipping, remained uncertain because if it was not degraded in the cells it could amass in the nuclei (38–40).

In the transplant area, we are currently trying to expand our surgical experience from SOT to SCT to repair tissues or parenchyma whose function has been impaired. SOT is a treatment more invasive than SCT. Solid organ substitution necessarily requires an allogeneic source, while SCT can exploit sane autologous cells or, in the treatment of genetic pathologies, *ex vivo*-modified autologous cells (41–43). Further, SOT

introduces potentially dangerous cells such as T lymphocytes or other immune effectors that could trigger GvHD (44). SOT could be the elective therapeutic choice to treat pathologies affecting single or double organs, while genetic and systemic diseases could be better treated with SCT. Cell graft permits to maintain the histological environment, such as stroma, nerves and vascular supply, unchanged. Unfortunately, in contrast to the SOT philosophy, cell seeding strategy requires specific timing to be effective because if the degenerative process would be progressed too far, 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 one of us outlined the clinical feasibility based on 20 yr of experimental background. However, a definite transplant protocol is still unsettled because of delay in performing Phase 1/2 clinical trials.

Various types of cells were investigated for cell therapy of muscular dystrophies (45). Among these cells, there were freshly isolated satellite cells, myoblasts proliferated *in vitro* from satellite cells, mesoangioblasts, pericytes and pluripotent cells called MDSCs.

Morgan et al. (46) recently demonstrated that myoblasts transplanted in an mdx muscle can be expanded following a muscle biopsy and retransplanted with success, suggesting that they function as satellite cells.

In a more recent clinical trial, our group transplanted allogeneic myoblasts into 1 cm³ of muscle with follow-up tacrolimus immunosuppression of one month. Normal allogeneic MT was able to restore the expression of Dys in up to 26% of the muscle fibers. A concurrent treatment in a 26-yr-old permitted us to restore the expression of Dys in 34.5% of the muscle fibers (47).

Huge improvement in MT outcomes could derive from genetically modified myoblasts being employed. Indeed, our group enhanced MT power by infection with lentiviral vector inducing overexpression of follistatin, protein stimulating muscular mass increase (48).

Discussion

New clinical trial of MT: patients' selection

The new clinical trial aims to transplant myoblasts in a single small muscle (the extensor carpi radialis) combined with six month of immunosuppression by tacrolimus and evaluate muscle strength improvement. The subjects for the study

will be recruited from young DMD patients, under 18 yr old. However, the ethic committee of Canada restricted protocol application to patients older than 18 yr old and seronegative for CMV. DMD patients older than 18 yr are in a very advanced stage of the disease:

- their muscle are largely infiltrated by fat and conjunctive tissue, so it is difficult to induce cellular fusion between donor and host myoblasts and restore muscular function;
- general health conditions required for the trial are frequently unsatisfied, because of frequent respiratory and cardiac compromise;
- forearm muscle movements are severely restricted and the strength assessments after MT are meaningfully limited.

In addition, 70% of adults in Canada are seropositive for CMV.

These are the reasons why the current guidelines preclude assessment on MT in Canada.

We think that if the muscular function is preserved or improved during the follow-up, it would be wise to transplant myoblasts into other muscles of the same patient to prevent further muscle failure.

One of the main objections to MT clinical trials concerns about side effects induced by immunosuppressive therapy and advantages deriving from the treatment on children. In the philosophy of solid organ transplant, only a life-threatening organ failure justifies the risks of transplant challenge. However, kidney failure is an exception. Dialysis and artificial kidney supports give the chance to avoid transplantation. Immunosuppressive drugs such as tacrolimus are routinely used in children, who are not in imminently life threatening, in renal transplantation (49–51). Hemodialysis makes worse life quality, but it permits to live out of the hospital, be autonomous in a lot of grooving actions and continue to experiencing some aspect of normal life (52–54).

Moreover, immunosuppression was used in trials treating children with type 1 diabetes, a disease that can be controlled with insulin administration (55).

We think that a similar exception should be made for DMD patients. Indeed, although their life can be extended to around 25 yr with respiratory assistance, their quality of life after 17 yr old progressively worsens: they become totally unable to move, to eat by themselves and to control their own electric wheel chair! Their parents have to feed them, change their diaper and turn them in their bed several times each night.

Although MT does not currently aim to prevent death, it may permit improvement in strength of several muscles permitting greater autonomy. It greatly justifies the risks associated with immunosuppression.

Tacrolimus immunosuppression in children

Tacrolimus is currently used in children for the treatment of various autoimmune diseases (56–58), many of which are less severe than DMD. During the last 10 yr, an adequate worldwide experience has been developed on tacrolimus (59) side effects. The list of centers where tacrolimus is being used for kidney transplantation is already quite long. The main adverse effects are increases in EBV and CMV infections (60); however, they are managed with ganciclovir or acyclovir (61). In a clinical trial, there were 13.7% lymphoproliferative diseases but 81.3% of them were controlled by drug treatment (62). A comparison between tacrolimus and Cy in 70 children aged 6.5 yr with a heart transplant showed a comparable nephrotoxicity between the two groups six yr after transplantation (63). An investigation showed 42 pediatric patients submitted to liver transplant and switched from Cy treatment to tacrolimus administration. At 16 months of follow-up, the main side effects were arterial hypertension (9.2%), liver toxicity (2.3%) and cosmetic damage (4.8%) (64). The hypertrophic cardiomyopathy was detected in five transplanted infants (three for small-bowel and two for liver) in association with tacrolimus administration. It was treated by reducing or stopping tacrolimus administration (65). One hundred and three patients less than 80 months old had kidney transplantation and treated with oral tacrolimus. Only three patients developed lymphoproliferative disease because of EBV infection. The incidence of type II diabetes was lower in children with tacrolimus than in those with Cy. Tacrolimus was better than Cy in the prevention of acute kidney rejection when used in combination with azathioprine and corticosteroids. Also graft survival at four yr improved (66). A study performed on 49 transplanted infants' hearts showed that the most frequent side effects were anemia, renal toxicity, hyperkalemia, gastrointestinal and allergic problems. Late deaths (1.4%) were mainly because of severe infection, coronary obstruction, lymphoproliferative disease and mitochondrial myopathy (67).

On the basis of this overview, the use of tacrolimus in children shows an acceptable therapeutic/side effects rate in organ transplant area.

Considering that DMD patients are otherwise healthy, tolerance to tacrolimus in this cohort should be even better. Furthermore, in the future, the immunosuppressive therapy used to prevent myoblast rejection in DMD patients might be modified to a multi-therapy approach with lower doses of each compound, excluding steroids. The availability of new immunosuppressive compounds such as sirolimus and everolimus, with specific anti-tumor capacity should also be evaluated to reduce the cancer risk. It could be developed as a sustained immunological tolerance to donor myoblasts and to new making hybrid muscle fibers, already achieved in mdx mice (68–70).

Can MT improve disease phenotype?

Our group has also obtained evidence supporting the beneficial effects of MT. When mdx mice that received MT were subjected to monthly eccentric exercise, damage was observed in Dys-negative fibers but not in the Dys-positive fibers (71). Probably, MT protected the muscle tissue of mdx mice from the mechanical stress, which triggers myofiber necrosis. Moreover, myoblasts grafted in a mouse model were able to form new muscle fibers (46).

Conclusion

We are living in the stem cell age, wherein in CNS diseases the goal to regenerate a neural network is strongly supported, while in heart pathology, myocardial regeneration after acute or chronic failure has been actively attempted. In the case of skeletal muscle diseases, the concept of repairing defective muscle fibers is possible by intra-dermal injections, possibly with a robotic arm, of a large number of myoblasts. In the future, MT should be carried out as soon as the first symptoms of muscular failure appear for two reasons: (i) a lower number of cells would be required because of under developed muscular mass and (ii) a better quality of viable muscular tissue will be colonized by the donor myoblasts.

There is evidenced that some of the transplanted myoblasts become satellite cells able to repair damaged muscle fibers. We believe that retransplantation of the same muscles will not be necessary because MT benefits will last a lifetime. We need to demonstrate the improvement of the contractile function and motility increase in the younger patients. Only such investigation will definitely confirm the feasibility of MT. We will struggle to make MT an easy, safe and effective standard procedure.

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