New therapies for muscular dystrophy: cautious optimism

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The quest for a therapy for muscular dystrophy has been the driving force behind the past 40 years of advances in this field. Numerous results, such as the identification of satellite cells and gene mutations that are responsible for most forms of dystrophies, advances in gene transfer and modification technology and, more recently, stem cells, have fueled hopes. However, administering corticosteroids still remains the only effective treatment available. Several recent advances have uncovered a diversity of possible therapeutic approaches, from pharmacological treatments to gene therapy (exon-skipping and adeno-associated viruses) and cell therapy with different types of newly identified stem cells. Importantly, a combination of these strategies might greatly enhance the possibility of successful therapy.

Muscular dystrophies are among the most difficult diseases to treat, even though the underlying molecular defects are now known. This is due to the fact that skeletal muscle is the most abundant tissue of the body and is composed of large multinucleated fibers, the nuclei of which have permanently lost the ability to divide. Consequently, any cell or gene replacement must restore proper gene expression in hundreds of millions of postmitotic nuclei, which are embedded in a highly structured cytoplasm and surrounded by a thick basal lamina. Similarly, most pharmacological trials must overcome the complex and partly unknown biochemical mechanism of fiber degeneration that involves pathways, such as calcium fluxes and protease activity, for which inhibitors are associated with high systemic toxicity. Nevertheless, the results that have been accumulated during the last few years have opened new perspectives for all these different approaches.

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The pharmacological approach

Several pharmacological strategies have been attempted to counteract the consequences of the dystrophic process, including protease inhibitors, calcium blockers and drugs that act on protein and lipid metabolism [5]. Few of these have produced promising results in animal models (almost exclusively mice) and even fewer have entered clinical trials, with little further success. Fiber degeneration is accompanied by a chronic inflammation (mainly macrophages and lymphocytes) that leads to sclerosis and a reduction of the vascular supply. This starts a vicious circle that reduces oxygen supply and increases the likelihood of degeneration for surviving and regenerated fibers [1]. Therefore, anti-inflammatory molecules appeared to be the logical therapeutic strategy and, for more than a decade, corticosteroids have represented the only pharmacological therapy with relatively modest but consistent beneficial consequences [6]. Recent data indicate that corticosteroid treatment in DMD can delay the loss of independent ambulation by 2 to 4 years, significantly reduce the risk of developing skeletal defects and delay the onset of respiratory and cardiac failure. However, their use is associated with significant side effects, such as weight gain and osteoporosis with the risk of bone fractures [7].

Recently, an old idea has been reconsidered on the basis of new results; muscle trophy is the balance between...
anabolic processes (the synthesis of contractile proteins and, to a minor extent, the proliferation and recruitment of progenitor cells) and catabolic processes (protein degradation due to calcium leakiness and the activation of proteases). It was reasoned that stimulating anabolic processes would counteract, or at least delay, muscle wasting. This was true for insulin growth factor 1 (IGF-1), which is a known muscle growth factor, and myostatin, a negative regulator of muscle growth. When mice over-expressing IGF-1 where crossed to mdx mice (a model for DMD), dystrophy was attenuated [8]. Similarly, when neutralizing antibodies against myostatin were systemically delivered to mdx mice, dystrophic mice showed a dramatic delay of muscle wasting [9]. Moreover, several other ‘booster’ proteins were identified that stimulated muscle regeneration and ameliorated dystrophy, such as integrin α7, acetyl-N-galactosamine transferase, and A disintegrin and metalloprotease 12 (ADAM12) (reviewed in [10]). Special attention was attracted by utrophin (a protein that is related to dystrophin), for which increased expression in mdx mice prevented the occurrence of pathology [11]. However the utrophin gene is large and, as is dystrophin, difficult to transfer. As an alternative approach, a high-throughput screen for small molecules that can upregulate utrophin synthesis was carried out but, so far, no new candidate drugs have been identified.

Blocking the proteasome in mdx mice causes the re-appearance at the membrane of many proteins of the dystrophin complex (except dystrophin itself), suggesting another method to counteract the muscle protein degradation [12]. Finally, the expression of nitric oxide synthase increased angiogenesis and also resulted in the amelioration of the mdx phenotype [13] by counteracting the reduced vascular supply that accompanies fiber degeneration, inflammation and sclerosis. Because muscular dystrophy defects result from the premature degradation of proteins that are important for muscle function and maintenance, these pharmacological agents might be invaluable for slowing down the progression of the disease, ameliorating the quality of life of the patient and, at the same time, increasing the chance of success for gene or cell therapy. It remains to be seen whether these strategies can be applied as a treatment, because the toxicity of these molecules must be assessed.

Gene therapy
The task of replacing a missing gene in all, or at least in a good proportion, of the post-mitotic nuclei of skeletal muscle is daunting. Furthermore, for DMD, the form of dystrophy that most needs a therapy, the gene to be replaced is the largest known, with a cDNA of 14 Kb [2]. Two successive waves of enthusiasm were generated by the use of adenoviral vectors that were successful in delivering dystrophin to a very large fraction of muscle fibers in newborn dystrophic mice [14]. The first generation of treatments, however, caused a strong immune reaction against the vector (which was not apparent in newborn animals, which were tolerant). The second generation of treatment, ‘gutted’ vectors, can accommodate the full-length cDNA for dystrophin, but do not carry genes encoding viral proteins. These induce a much weaker immune reaction, but have to cross the basal lamina of muscle fibers and the efficiency of transduction is greatly reduced in juvenile and adult animals [15–16].

Adeno-associated viral (AAV) vectors are derived from a non-pathogenic replication-deficient virus with a small (~4.7-kb) single-stranded DNA genome. They appear to be more efficient for transducing adult fibers (owing to their smaller size) [17], especially if delivered systemically together with factors that increase vascular permeability [18–19]; a clinical trial using these vectors is ongoing. They cannot accommodate the full-length dystrophin cDNA, but a truncated version (micro-dystrophin) that gives good functional rescue when replacing dystrophin in transgenic mdx mice [20]. Moreover, they can accommodate the full-length sarcoglycan cDNAs, the proteins that are mutated in several forms of limb girdle muscular dystrophies [1].

An alternative strategy for gene therapy involves ‘exon skipping’. This molecular strategy prevents the transcription of the exon containing the mutation. Skipping can be achieved through oligonucleotides or by small RNAs that hybridize with the donor and/or acceptor sites of the mutated exon, causing its exclusion from the otherwise intact transcript. Because the skipped exon usually does not encode a functionally essential domain, the resulting protein is shorter but functional. Despite the fact that the oligonucleotides appear to function for only a short period in vitro, they are much more stable in muscle fibers in vivo and, recently, long-term correction of dystrophy in mdx mice was achieved by a single injection of oligonucleotides [21]. Based on this finding, several clinical trials in patients have been planned, and one in UK has now been funded. Other gene-therapy approaches, such as plasmid DNA injection or DNA–RNA chimeric oligonucleotides, which currently appear to be less efficient, have been reviewed previously [22–23].

Cell therapy
The identification of satellite cells in 1961 [3] offered the first hope for treating muscular dystrophy with cells that can make new muscle. Since the beginning, two alternatives appeared: (i) using cells obtained from a healthy donor, which express the normal copy of the mutated gene but induce an immune rejection unless the patient is permanently immune suppressed; or (ii) using cells obtained from the patient, which do not require immune suppression but must be ‘genetically corrected’ in vitro (to restore the expression of the mutated protein). This latter task was made possible (although far from easy) by the cloning of the genes that result in muscular dystrophy. Satellite cells and cell lines derived from them have been used since the late 1970s, mainly through intra-muscular injection. A first pivotal study involved the injection of wild-type myoblasts (from the immortal myogenic cell line C2C12) into mdx mice and resulted in the conversion of muscles from dystrophin-negative to dystrophin-positive [24]. This study led to several clinical trials in the early 1980s that failed for several reasons, the most important of which were the poor survival and the very limited migratory capacity of injected donor cells, together with the immune response of the patient that was not
suppressed at that time. Myogenic cell transplantation was continued and optimized in few laboratories in preclinical studies. Very recently, a novel clinical trial with partially matched donor cells and immune suppression showed the reconstitution of up to 11% of dystrophin-positive fibers in the area of injection [25].

The major problem still faced by this approach is the lack of dispersion of donor cells, which remain in the area of injection, making it difficult to reach an even distribution within the whole muscle. This might be overcome by using blood-borne stem or progenitor cells. This perspective became theoretically possible in the late 1990s, with the demonstration of cells in the bone marrow that could contribute to muscle regeneration following bone marrow transplantation [26]. The possible systemic delivery of circulating cells was the obvious choice over satellite cells that cannot cross the endothelial layer. It was subsequently reported that a fraction of bone marrow cells, the SP (side population, which are characterized by the ability to exclude dye as a result of the presence of a multi-drug exclusion pump that is typical of stem cells) would give rise to dystrophin-positive fibers in the mdx mouse following bone marrow transplantation [27]; in both studies, the extent of colonization by donor cells was very small, far from any hope of clinical benefit. During the following years, many reports appeared describing the trans-differentiation of bone marrow cells into embryologically unrelated cell types, such as hepatocytes, neurons, cardiomyocytes and epithelia. Controversy soon arose on the significance of these data, which have been interpreted as methodological artifacts or rare events of cell fusion rather than signal-mediated changes in cell fate (for a thoughtful review, see [28]). Leaving aside the possibility of artifacts, cell fusion would result in the exposure of the donor-cell nucleus to the host-cell transcription factors and, for muscle, the dominant effect of MyoD would soon activate muscle genes in the donor cells. Although this might not be a major problem in terms of future clinical applications (especially considering that muscle cells are multinucleated) the rarity of the event and our ignorance on the underlying mechanism make this process a distant future clinical application.

During the last two years, however, several reports have convincingly demonstrated that bone marrow SP cells can be recruited to dystrophic or regenerating muscle and can differentiate into skeletal muscle cells upon exposure to differentiating muscle cells or in response to Wnt molecules that are secreted by recruiting cells; moreover, a fraction of SP cells localizes to a position (between the basal lamina and the sarcolemma) that is typical of satellite cells and expresses markers of satellite cells [29–31]. Two independent groups showed that the progeny of a single SP cell can reconstitute the hematopoietic system of a mouse following bone marrow transplant and can also contribute cells to regenerating muscle [32,33]. Finally, a recent paper showed that it is possible to transduce SP cells from a dystrophic mouse with a lentiviral vector expressing human micro-dystrophin, and reconstitute a few dystrophic fibers with the human protein following intra-vascular delivery [34]. Interestingly, cells expressing the hematopoietic marker AC133 that can differentiate into dystrophin-positive fibers in vivo are present in the human circulation, suggesting that strategies developed in murine models might later be transferred to patients [35].

Currently, two questions remain unanswered: (i) whether SP cells are recruited to myogenesis through signal-induced myogenic commitment (including differentiation into satellite cells) or through the fusion of a myeloid intermediate cell with host muscle fibers (or eventually both) and (ii) whether SP cells are the only stem or progenitor cell associated with the hemo-vascular system that posses myogenic potential. In favor of signal-mediated commitment is the induction by Wnt molecules, similar to what happens in embryonic myogenesis, and the identification of donor SP-derived cells expressing satellite cell markers in a location typical of satellite cells [29–32]; in favor of fusion is the expression of a myelomonocytic marker in the population with myogenic capacity and the failure to detect donor-derived satellite cells in vivo and in culture [33]. Evidence that SP cells are not the only mesoderm progenitor that can differentiate into skeletal muscle comes from several studies showing that different CD45 (a pan-myeloid marker) negative cells, such as multipotent adult progenitors (MAPs) [36], mesoangioblasts [37] or muscle-derived stem cells (MDSCs) [38], can differentiate into skeletal myotubes in vitro and in vivo when delivered to regenerating or dystrophic muscle (Figure 1). The expression of CD45 in SP cells clearly defines the hematopoietic nature of these cells; the lack of CD45 in these other types of stem cells, which are generally associated with the vascular niche in bone marrow, skeletal muscle [38,39] or other tissues [40], identifies them as non-hematopoietic and probably belonging to the endothelial or pericyte lineages [41].

Notably, in at least one case in which wild-type or dystrophic genetically corrected mesoangioblasts were delivered intra-arterially to dystrophic muscle of z-sarcoglycan knockout mice (a model for limb girdle muscular dystrophy), it resulted in a dramatic functional amelioration of the dystrophic phenotype [42]. In principle, to optimize the efficacy of stem-cell therapy for muscular dystrophy, it will be necessary: (i) to isolate cells from an easily accessible anatomical site; (ii) to expand them in vitro without the loss of stem-cell properties; (iii) to efficiently transduce them with viral vectors (such as lentiviral vectors, which are by far the most efficient integrating vectors but have not yet been approved for use in patients); and (iv) to facilitate homing to diseased muscle through circulatory routes by using molecules that can recruit them, such as HMGB1 (high-mobility group B1) [43]. SP cells are still difficult to expand in vitro but recent advances for the expansion in vitro of hematopoietic stem cells should hopefully solve this question [44]. CD45-negative stem cells have not been systematically tested for their ability to rescue dystrophic muscle by intra-vascular delivery (Table 1), with the exception of mouse mesoangioblasts, for which the human counterparts are still being characterized.

Concluding remarks
The current advances that have been made in pre-clinical research in the field of muscular dystrophy justify a
cautious optimism, more so than only a few years ago [45]. Results from clinical trials will be available in the coming months: it would not be wise to expect spectacular results. It is, however, realistic to expect that some beneficial effect will be observed in some of the ongoing studies and this ought to be possible without any major (at least short term) toxicity. This would justify the planning of further trials that could incorporate the predicted results from ongoing experimental work. New generations of viral vectors, improved methods for efficient and long-lasting exon skipping, and increasing knowledge about the various types of stem cells (resulting in more efficient ways to manipulate them) should be the basis for the next generation of trials, all of which might benefit from the design of combined pharmacological therapies. In turn, these will need to be tested on patients independently from gene or cell therapy to select the most efficacious and least toxic for successive combined therapy. It is probable that muscular dystrophies, which present such a complex pathogenesis, will only be defeated by a combined effort that is aimed to replace or correct the mutated gene product and simultaneously counteract the devastating consequences of the primary mutation on muscle structure and function.

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References

Table 1. The phenotype of different stem cells and their potential to contribute to muscle regeneration in dystrophic muscle

<table>
<thead>
<tr>
<th>Stem cell</th>
<th>Source</th>
<th>Growth in vitro</th>
<th>Homing to diseased muscle?</th>
<th>Myogenic differentiation induced by:</th>
<th>Effect in primary myopathy</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic stem cells (SP)</td>
<td>Bone marrow</td>
<td>Limited</td>
<td>Yes</td>
<td>Co-culture with myogenic cells; Wnts 5'-Aza-cytidine</td>
<td>Modest (&lt;1%)</td>
<td>[27,29–33]</td>
</tr>
<tr>
<td>Multipotent adult progenitors</td>
<td>Bone marrow</td>
<td>Yes</td>
<td>Unknown</td>
<td>Co-culture with myogenic cells</td>
<td>Unknown</td>
<td>[36]</td>
</tr>
<tr>
<td>Mesoangioblasts</td>
<td>Small vessels</td>
<td>Yes</td>
<td>Yes</td>
<td>Co-culture with myogenic cells</td>
<td>Good (~30% of muscles downstream injected artery)</td>
<td>[37]</td>
</tr>
<tr>
<td>Endothelial myogenic progenitors</td>
<td>Muscle vessels</td>
<td>Limited</td>
<td>Unknown</td>
<td>Co-culture with myogenic cells</td>
<td>Unknown</td>
<td>[39]</td>
</tr>
<tr>
<td>Muscle-derived stem cells</td>
<td>Skeletal muscle</td>
<td>Yes</td>
<td>Yes</td>
<td>Low serum; standard myogenic culture 5'-Aza-cytidine</td>
<td>Good (&gt;90% in injected area)</td>
<td>[38]</td>
</tr>
<tr>
<td>Sinovial muscle stem cells</td>
<td>Sinovial membranes</td>
<td>Yes</td>
<td>Yes</td>
<td>Low serum; standard myogenic culture 5'-Aza-cytidine</td>
<td>Modest (&lt;1%)</td>
<td>[40]</td>
</tr>
</tbody>
</table>