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The new frontier in muscular dystrophy research: booster genes

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ABSTRACT

More than 30 different forms of muscular dystrophy (MD) have been molecularly characterized and can be diagnosed, but progress toward treatment has been slow. Gene replacement therapy has met with great difficulty because of the large size of the defective genes and because of difficulties in delivering a gene to all muscle groups. Cell replacement therapy has also been difficult to realize. Will it even be possible to design specific therapy protocols for all MDs? Or is a more realistic goal to treat some of the secondary manifestations that are common to several forms of MD, such as membrane instability, necrosis, and inflammation, and to promote regeneration? As reviewed here, enhanced expression of a range of proteins provides a boost for degenerating dystrophic muscle in mouse models. Expression of a mini-agrin promotes basement membrane formation instead of laminin α2; integrin α7, GalNac transferase, and ADAM12 promote cell adhesion and muscle stability in the absence of dystrophin; calpastatin prevents muscle necrosis; and nitric oxide synthase prevents inflammation. ADAM12, IGF-I, and myostatin blockade promote regeneration and reduce fibrosis. One can envision numerous other candidate booster genes which encode proteins that promote survival and/or regeneration of the compromised muscle or proteins that affect post-translational modifications of critical proteins. Finally, fibrosis, which is the curse of many human diseases, may also be attacked. Once the mechanisms of the boosters are better understood, drugs may be developed to provide the boost to muscle. Some of the experiences in models of muscular dystrophy may inspire new approaches in other genetic degenerative diseases as well.—Engvall, E., Wewer, U. M. The new frontier in muscular dystrophy research: booster genes. *FASEB J.* 17, 1579–1584 (2003)

Spectacular progress has been made in understanding the molecular/genetic basis of muscular dystrophy (MD). Beginning with the discovery of the dystrophin gene and protein 15 years ago (1, 2), more than 30 forms of MD can now be accurately identified by using genetic or immunochemical methods (3, 4). We have learned from the molecular basis of the MDs that structure is of utmost importance in the maintenance of muscle function. With this knowledge has come the realization that the structural defects in MD will be very difficult to repair. Many of the proteins defective in MDs are very large and complex and form large structural networks. Faulty muscle structure caused by the absence of extracellular or intracellular structural proteins results in cell membrane instability, initiating a cascade of increasingly more damaging events: calcium influx, apoptosis and necrosis, inflammation, fibrosis, and replacement of muscle with connective tissue and fat. Whole cell replacement, gene

replacement, and gene repair have been explored to deal with the primary defects in MD (5–9). However, these methods are still far from being applicable in the clinic; even when available, they may not always be the best solution for all forms of MDs.

CLASSICAL GENE THERAPY FOR MUSCULAR DYSTROPHY

The principle of gene therapy for MD is simple: a muscle with a defective gene is supplied with the normal version of the gene. Muscle is indeed a tissue that readily expresses such transfected or transduced genes. However, gene therapy is difficult to accomplish in practice. Current technology does not allow efficient distribution of genes to many muscle groups. The delivery of genes to even a small number of muscle fibers in the ~500 muscle groups in the body is indeed a daunting task. Such delivery will ultimately require a number of new technologies, such as distribution of genes via the circulation and the uptake of genes specifically in skeletal muscle cells and not in other cells. Another problem to be solved if efficient distribution of genes is accomplished is the possible immune reaction to a protein, which was previously absent. Finally, insertion of the replacement gene in the genome may introduce new mutations in other genes (10).

While gene therapy is still far from being applicable in the clinic, great progress has been made in understanding the molecular biology and function of dystrophin. Smaller and smaller variants of dystrophin have been engineered and shown to be capable of functionally replacing the intact protein. These experiments culminated in the demonstration of a relatively well-functioning “microdystrophin” of 167 kDa, less than half the size of the full-size protein (11, 12).

COMPENSATING GENE DEFECTS WITH BOOSTER GENES

Investigators have explored a different form of gene therapy: expression of “booster genes,” aimed at alleviating the secondary defects in MD rather than the primary ones. The products of “booster genes” support the muscle to cope better with the otherwise damaging steps of the path to muscular dystrophy. **Figure** 1 shows the booster genes reviewed here. Other potential booster genes could be similarly tested. For example, it may be possible to reduce fibrosis with transforming growth factor-β inhibitors (13) and to direct mesenchymal stem cells to differentiate into muscle rather than fat (14–16). Two mouse models have been used in these studies—the dystrophin-deficient *mdx* mouse and the laminin α2-deficient *dy* mouse—representing common forms of MD in humans. The details of the transgenes used are summarized in **Table** 1 and are described below.

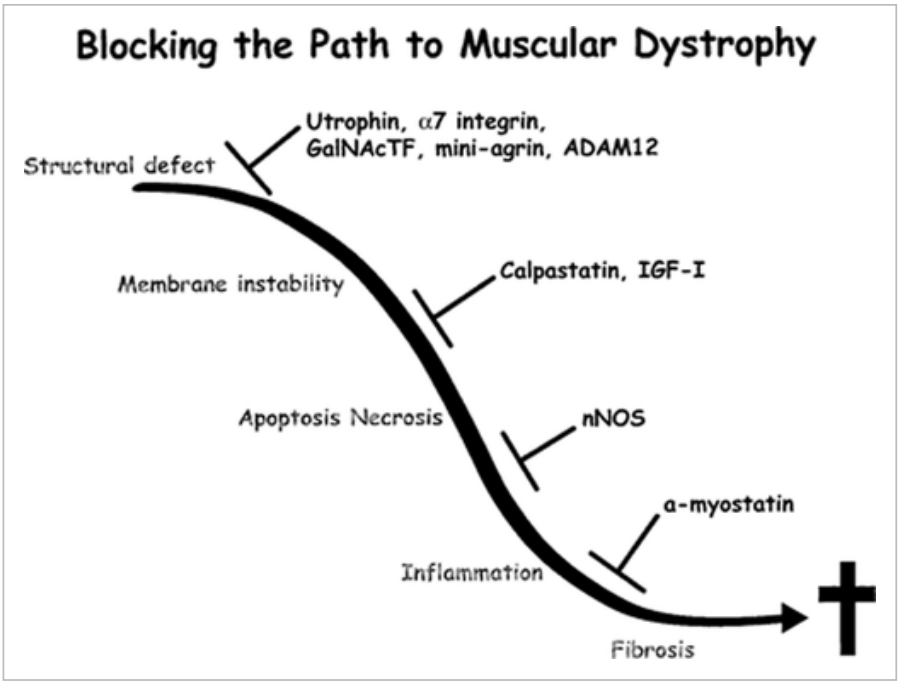

Figure 1

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Booster genes to prevent or stop the progression of muscular dystrophy. The primary gene defect, which often results in a structural defect of muscle, initiates the path toward muscular dystrophy and results in increasingly more damaging secondary events. The products of tested booster genes help the muscle resist degeneration by preventing, reversing, or counteracting the different damaging events.

Table 1. Booster genes that alleviate MD in mice

Mouse strain	Form of muscular dystrophy	Transgene/treatment	Promoter	Reference
mdx/utr− /−	Dystrophin deficiency	Integrin α7 tg[a]	MCK	17
mdx		n-NOS tg	Skeletal actin	31
mdx		IGF-I tg	Myosin light chain	32
mdx		ADAM12 tg	MCK	24
mdx		GalNAc transferase tg	Skeletal actin	19
mdx		Calpastatin tg	Skeletal actin	29
mdx		Myostatin knockout		36
		Anti-myostatin		35
dyW	Laminin a2 deficiency	Laminin α2 tg	MCK	43
dyW		Mini-agrin tg	MCK	39

a Transgene.

Integrin α7β1

There are two major cell adhesion complexes in muscle: the dystrophin-associated glycoproteins (DAGs) and complexes associated with integrin α7β1. When dystrophin is missing, the DAGs are reduced or absent. Transgenic overexpression of integrin α7in *mdx/utr−/−* mice at a modest two- to threefold level was enough to extend significantly the life span of the mice (17). Muscle pathology was attenuated at all ages. Curiously, the integrin transgene did not prevent the initial cycle of necrosis that occurs at ~4 wk in the *mdx/utr− /−* mice, but seemed to stabilize the muscle once postnecrosis regeneration had occurred. Perhaps higher expression or a promoter active earlier in development would provide a high enough expression of integrin α7 to also protect against the early muscle necrosis. The likely mechanism of action of integrin overexpression is that the integrin partially substitutes for adhesion mediated through the DAG complex.

GalNAc transferase

Dystroglycan needs to be extensively glycosylated for it to bind efficiently to laminin (18). Overexpression of a GalNAc (N-acetyl galactosamine) transferase had a dramatic beneficial effect on *mdx* muscle, resulting in near-normal microscopic appearance and minimal degeneration. Dystroglycan was modified with terminal GalNac and was highly up-regulated together with utrophin in transgenic mice. It was proposed that the additional glycosylation of dystroglycan may have strengthened the link to laminin in the extracellular matrix (19) and that the increased expression of utrophin compensated for the lack of dystrophin. The benefit of the overexpression of the transferase is at first surprising, given that overexpression of the enzyme in wild-type mice had a negative effect on muscle development, as was apparent from the small and immature muscle fibers in such mice (20). The power of glycosylation these experiments show is also evident from the discovery of muscular dystrophies that are caused by abnormal glycosylation (21). Perhaps other post-translational modifications in proteins, such as phosphorylation, can be modulated and used to support and maintain muscle in MD.

ADAM12

Adam12 belongs to a family of more than 30 members that have metalloprotease and integrin binding activity. ADAM12 (A disintegrin and metalloprotease) is highly expressed in muscle development and regeneration, but not in adult muscle (22, 14, 23). The specific roles of ADAM12 in skeletal muscle are not known. To explore the potential role of ADAM12 in muscle, we engineered *mdx* mice with continuous skeletal muscle expression of ADAM12 (24). A significant reduction of several aspects of *mdx* muscle pathology was seen. Some of the ADAM12 effects may be due to its cell adhesion-promoting activities and may be related to its binding to syndecans and integrins (25, 26). The ability of ADAM12 to enhance the presence of utrophin and integrin α7 at the cell membrane may also be important (B. Moghadaszadeh et al., unpublished results). Moreover, as a metalloprotease (27), ADAM12 may sequester and process growth factors that affect muscle regeneration (see below; 28)

Calpastatin

The membrane defect in dystrophic *mdx* muscle results in influx of proteins and ions into the cell. The influx of calcium may lead to a cascade of autoproteolysis. Direct evidence for the importance of the calcium-activated calpain proteases in muscle degeneration comes from recent experiments with transgenic over-expression of calpastatin in *mdx* mice (29). Calpastatin is a specific inhibitor of m- and μ-calpains. Overexpression of calpastatin in muscle inhibited the activity of endogenous calpains in *mdx* mice and greatly reduced muscle necrosis.

Necrosis was reduced even though membrane leakage was not. Calpastatin overexpression is a particularly striking example of the significant benefit that can be obtained from targeting downstream effects of a gene defect without addressing the primary defect.

Nitric oxide synthase

The groups of Tidball and Spencer have pointed out the potentially damaging effect of excessive muscle inflammation in dystrophin deficiency (30). Neuronal nitric oxide synthase (nNOS) is normally localized to the membrane of muscle cells, but in dystrophin deficiency, nNOS is not membrane associated, and production of nitric oxide is reduced. Overexpression of an nNOS transgene normalized nitric oxide levels in *mdx* muscle, with resultant reduction in necrosis and release of creatine kinase into serum (31). The presence of fewer macrophages and immune cells in *mdx-nNOS* muscle was in agreement with an anti-inflammatory effect of nNOS overexpression. Depletion of macrophages in *mdx* mice via systemic administration of a monoclonal antibody had a similar beneficial effect on the dystrophic muscle.

IGF-I

Regeneration of muscle fibers may compensate for degeneration at early stages of MD. However, over time, regeneration is compromised by several factors, including the depletion of precursor cells and the pathological environment in the muscle. Two potential boosters of regeneration have been tested in transgenic mice: ADAM12 and insulin-like growth factor-I (IGF-I). Continuous overexpression of ADAM12 in the muscle of wild-type mice greatly accelerated muscle regeneration after injury (B. Moghadaszadeh et al., unpublished results). Thus, one of the effects of ADAM12 in *mdx* mice may be enhanced regeneration (24). IGF-I plays many roles in normal myogenesis including promotion of proliferation, differentiation, and survival of muscle cells. Barton et al. (32) showed that moderate overexpression of IGF-I in *mdx* muscle resulted in decreased necrosis and fibrosis and increased muscle mass and strength. The phosphorylation of the antiapoptotic protein Akt was dramatically and specifically increased in the *mdx/IGF-I* muscle, suggesting that IGF-I had a particularly positive effect on cell survival in dystrophin deficiency. Consequently, IGF-I not only promotes regeneration but also prolongs the life of mature muscle fibers.

Myostatin

Myostatin is a negative regulator of muscle formation. Animals with mutations in the myostatin gene have both muscle hyperplasia and hypertrophy and less fat and connective tissue in muscle (33). Administration or overexpression of myostatin induced muscle atrophy (34). It may then be beneficial to block, remove, or reduce myostatin to promote regeneration and reduce fibrosis in MD. Bogdanovich et al. (35) blocked myostatin in young *mdx* mice by intraperitoneal injections of a neutralizing antibody. Pathology, including necrosis and increased levels of serum creatine kinase, was reduced in the anti-myostatin-treated mice compared with untreated *mdx* mice. Wagner et al. (36) used a different approach to remove myostatin. They generated myostatin-deficient *mdx* mice by breeding the *mdx* mice to myostatin null mice. They found that the myostatin-deficient mice had increased muscle mass and muscle strength. On the other hand, they did not find a significant reduction in necrosis or inflammation. However, the fibrosis and fatty replacement seen in older *mdx* mice was significantly reduced in the myostatin-deficient *mdx* mice. Both studies thus concluded that some benefit is obtained by removing myostatin. The two studies used quite different methods and analyzed mice at different ages and do not agree on the type of benefit. If the hypothesis is that reducing myostatin will increase muscle regeneration, perhaps a different model than the *mdx* mouse is needed, as *mdx* mice already have efficient muscle regeneration. The attractive aspect of a potential future anti-myostatin therapy is that several myostatin

inhibitors or blockers could be used for systemic administration, including the myostatin prodomain, follistatin, and soluble receptors (37, 38).

A structural booster gene: mini-agrin

Moll et al. (39) designed a novel way to increase cell adhesion in muscle. An agrin minigene was used to enhance cell adhesion and basement membrane formation in laminin α2 deficiency. The 120 kDa miniagrin was constructed from the amino-terminal laminin binding and the carboxyl-terminal dystroglycan binding domains of the much larger intact agrin molecule. Laminin α4 is up-regulated in laminin α2 deficiency but is not able to compensate for laminin α2, because it cannot form a basement membrane and does not bind to dystroglycan. Laminin α5 is also increased but not in sufficient amounts. The aim was to cross-link dystroglycan on the cell with the laminin α5 in the matrix via mini-agrin and thereby retain more laminin α5. Indeed, the expression of mini-agrin up-regulated laminin α5 and prevented the structural and functional deficits seen in laminin α2 deficiency. The basement membranes were even structurally intact in these mice. The mice were active and long-lived, with near-normal muscle strength. It may be possible in the future to use similar de novo protein design to generate other small artificial proteins with binding sites for selected target molecules. If such artificial proteins are constructed from existing protein segments, immunogenicity should not pose a problem.

WHAT IS THE THERAPEUTIC RANGE OF BOOSTER GENES?

Can ectopic gene expression be dangerous? At first it appeared that ectopic expression of various genes was harmless, even when expression was at nonphysiological levels. Overexpression of dystrophin and utrophin was reported to have no deleterious effect on normal muscle (40, 41). Similarly, a 50-fold overexpression of nNOS in normal skeletal muscle did not result in excess nitric oxide production or muscle pathology (31). Apparently, dysregulation of the corresponding genes is not toxic. However, more recent results indicate that this is not always the case. The regulation of other genes, such as growth factor genes, may require more stringent control of gene expression. Low-level expression of IGF-I had no adverse effects (32), whereas high-level expression resulted in elevated circulating IGF-I and cardiac hypertrophy (42). Overexpression of a human laminin α2 transgene in the muscles of laminin-deficient or wild-type mice resulted in centrally located nuclei, indicative of regeneration in response to injury (43). In other cases, overexpression of trans-genes had different effects on normal and pathological muscle. Although overexpression of ADAM12 and Gal-Nac transferase was beneficial to dystrophic muscle, the overexpression of ADAM12 in normal muscle resulted in some remodeling of the muscle (B. Moghadaszadeh et al., unpublished results), and overexpression of GalNAc transferase resulted in rather severe pathology with reduced muscle mass (20). Finally, overexpression of γ-sarcoglycan in normal muscle produced muscle necrosis that was almost as severe as that in γ-sarcoglycan-deficient mice (44). It is also important that an appropriate splice form of the protein is expressed (45). Another issue is whether a transgene that can prevent or delay disease can also alleviate preexisting disease. Recently, expression of utrophin was regulated by the tet-off system in *mdx* mice (46). Overall, the site, level, and timing of gene expression can have significant effects on muscle function and may need to be carefully controlled. In the future, the potential effect of different genetic backgrounds must be understood, and added emphasis may have to be placed on regulation of potentially therapeutic genes.

CAN THE EFFECTS OF BOOSTER GENES BE REPRODUCED WITH DRUG TREATMENTS?

At this time we know very little about the mechanisms by which the booster genes exert their effects on dystrophic muscle. When these mechanisms are better understood, it may be possible to develop small molecule

inhibitors, agonists, and antagonists to mimic some of the activities of the products of the booster genes. For example, if the main function of n-NOS in the dystrophic muscle is to inhibit inflammation, then many existing drugs may help. In fact, the only current treatment that has any benefit in human dystrophin deficiency is prednisone (47). In the future, the lack of glycosyl transferase activity may be counteracted by excess sugars (48, 49) and the function of calpastatin may be mimicked by chemical calpain inhibitors (50). Overall, the experience gained by the expression of booster genes in mouse models with muscular dystrophy will certainly increase our understanding not only of the function of muscle, but also of the normal function of the booster genes and the pathogenesis of muscular dystrophy. We may learn what will be beneficial to combat cell degeneration in other diseases than muscular dystrophy, such as the skin blistering diseases and Alzheimer disease, which have intrinsic cellular defects as well as accompanying and destructive inflammatory components.

CONCLUSIONS

The 30 different forms of MD are waiting for treatments. Will it be possible to design specific gene therapy protocols for all? It may be more realistic to treat some of the secondary manifestations that are common to several forms of MD, such as membrane instability, inflammation, and necrosis, and to promote regeneration. As reviewed here, this can be accomplished in mouse models by ectopic expression of a range of proteins that provide a boost for degenerating dystrophic muscle. We can envision numerous other candidate booster genes that encode proteins that promote survival and/or regeneration of the compromised muscle or affect post-translational modifications of proteins. Given that glycosylation can have dramatic effects on dystroglycan and muscle stability and survival, it is possible that phosphorylation/dephosphorylation of signaling proteins in muscle can also be used to boost dystrophic muscle. Finally, fibrosis, which is the curse of many human diseases, may also be attacked.

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