Dystrophin Levels Required for Genetic Correction of Duchenne Muscular Dystrophy

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Abstract

Correction of the muscle pathology in Duchenne muscular dystrophy (DMD) could theoretically be achieved if methods are developed to produce functional versions of the dystrophin protein in muscle tissue. While a variety of approaches are currently being tested to achieve this aim, we have explored the feasibility of correcting the dystrophic pathology using transgenic mouse model systems. By generating transgenic mice expressing various levels of the muscle isoform of dystrophin in striated muscle we have determined how much dystrophin is needed to prevent the dystrophic pathology. Similarly, we have also explored the use of internally truncated dystrophins that would be of a size that could be inserted into a variety of viral vectors being considered for use in gene therapy protocols. Finally, we have also explored the percentage of muscle fibers that would need to be converted to a dystrophin positive phenotype to achieve a substantial correction of the pathology. The results indicate that a majority of fibers must accumulate approximately 20% of wild-type levels of dystrophin for a significant correction of the muscle pathology.

Key words: dystrophin, *mdx* mice, gene therapy, transgenic mice.

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Several approaches being developed to treat Duchenne muscular dystrophy (DMD) seek to restore production of the dystrophin protein, or a functional homologue such as utrophin [4, 16, 22]. The recessive nature of DMD indicates that a treatment strategy need only provide new dystrophin, without concern for damaging effects of endogenous mutant dystrophins. Indeed, a variety of experiments using transgenic animals have shown that high levels of mutant dystrophins do not by themselves lead to deleterious side effects [3]. Similarly, high levels of wild-type dystrophin or of truncated utrophin molecules lead to a correction of the myopathic phenotype in *mdx* mice, an animal model for DMD [17, 19]. As a result it is reasonable to believe that any method able to produce therapeutic quantities of dystrophin or utrophin would result in a treatment for DMD. A variety of methods are being tested to achieve this aim, including direct gene transfer, adapting viruses to deliver therapeutic mini-genes, using myoblasts to deliver wild-type dystrophin genes, or using drugs to upregulate production of utrophin [4, 7, 11, 12, 15, 21, 23]. The marginal success of these efforts to date is indicative of the enormous obstacles that must be overcome to deliver even small amounts of a therapeutic protein to muscle. One approach to determining the quantities of dystrophin or utrophin needed for successful therapy is to express various amounts of dystrophin in muscles of *mdx* mice. This approach can reveal the minimal levels of dystrophin needed for correction of the disease, the percent of muscle fibers that must be restored to a dystrophin positive phenotype, and when during development the dystrophin must be produced. We have addressed these questions using transgenic mice that express various amounts of dystrophin. The results provide a reference for the clinical end points that must be achieved for treatment of DMD.

Methods

Transgenic mouse lines were generated by injection of various dystrophin expression vectors into fertilized mouse eggs produced from a cross between C57B1/6 and SJL/J mice essentially as described [10]. F_0 mice carrying the dystrophin transgene were identified by polymerase chain reaction analysis of tail DNA, and these animals were crossed for several generations onto a C57B1/1 0*mdx* background. Animals between 3-6 months of age were used for analysis of serum levels of muscle creatine kinase (MCK). Fibers with central nuclei were counted from fixed sections of diaphragm muscle strips from at least two independent animals. At least 1000 fibers were counted from 3-4 separate strips in each animal. Force and power generating capacities of muscles were measured as described previously [13]. Dystrophin levels were determined by Western blot analysis and densitometry as described elsewhere

[17]. Immunofluorescent analysis of dystrophin expression was determined using antisera raised against the C-terminal domain of murine dystrophin [5].

Results

Overexpression of dystrophin

A number of the transgenic mouse lines generated in our laboratory display significantly higher than wild-type levels of normal or truncated dystrophins in striated muscles. One line of mice was observed to express approximately 50 times greater than wild-type levels of the full-length dystrophin [4]. Several additional lines expressed greater than 10-20 times the control levels of a dystrophin molécule lacking 16 of the 24 spectrin-like repeats in the central rod domain of the protein. This latter molécule is based on the mutation in a mildly affected Becker muscular dystrophy patient with a deletion of exons 17-48 [6]. In none of thèse animais were any deleterious side effects observed from the Overexpression of dystrophin. For example, despite thèse elevated dystrophin levels no muscle pathology was observed on the *mdx* mouse background [4]. In addition, when expressed on a wild-type background, no significant différences were observed between sérum creatine kinase levels or muscle force and power generating capacities of the control and transgenic mice (Figure 1). While it is unlikely that such high levels of dystrophin could be achieved in a clinical gene transfer protocol, it is likely that extremely high dystrophin levels could accumulate in small régions of muscle fibers near sites of delivery vector entry (virus, myoblast or other vehicle) into the muscles. The available data suggests focal high concentrations of dystrophin should not cause adverse effects.

Indeed, transgenic animais that express very high levels of dystrophin in a mosaic pattern do not display adverse effects, but display a partial amélioration of the dystrophic phenotype [17].

Underexpression of dystrophin

A more significant concern in gene transfer protocols may be the minimal amount of dystrophin needed to correct the genetic deficiency. Many of the transgenic lines we have generated accumulate less than wild-type levels of various dystrophin proteins. Typically we have observed two types of transgene expression levels in thèse animais. Most of the lines of mice expressed relatively uniform levels of dystrophin in différent muscle fibers. However, approximately one-third of the lines express dystrophin in a variable or mosaic pattern (see below).

In a detailed comparison of the dystrophic pathology in différent lines of transgenic *mdx* mice we observed that animais with at least 20% of the wild-type dystrophin levels displayed few détectable signs of dystrophy. When the transgene encoded the full-length muscle isoform of dystrophin no pathology was detected as measured by sérum CK levels, the percentage of fibers with centrally located nuclei [indicative of a prior cycle(s) of régénération], or the ability to generate force (Figure 2A and B

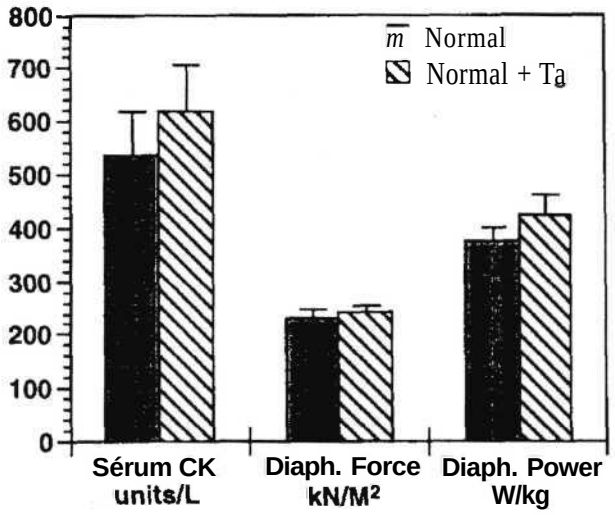

Figure 1. Lack of toxicity due to overexpression of dystrophin in wild-type mice. Wild-type transgenic mice expressing full length mouse muscle dystrophin from the -3.3 kb mouse muscle creatine kinase (MCK) enhancer/promoter élément were analyzed for sérum levels of MCK protein, as well as the force (kilonewtons per square meter -kN/M²) and power (watts per kilogram - W/Kg) generating capacity of diaphragm (Diaph.) strips. Thèse transgenic animais express approximately 50 fold higher than control values of dystrophin in striated muscle [4]. No significant différences were observed between transgenic and control (C57Bl/10) animais. Shown are absolute values plus the standard error of the mean (N = 4-6 for each assay).

[17]). Animais with less than 20% of wild-type levels displayed intermediate signs of dystrophy, suggesting that even small amounts of dystrophin can help prevent dystrophic processes from arising. However, at least 20% of the wild-type levels are necessary for a complète prévention of disease.

When a truncated dystrophin containing the exon 17-48 deletion was expressed, signs of fiber régénération were detected at low levels (Figure 2C). Interestingly, thèse dystrophic features were also présent even in those animais expressing higher than normal levels of this truncated molécule, indicating that this truncated protein is unable to alleviate all dystrophic features. Nonetheless, the lack of fibrosis coupled with the essentially normal force development in muscles of mice with approximately wild-type levels of the truncated dystrophin indicates that smaller protein could be useful in a clinical setting if vectors able to deliver the full-length 430 kDa dystrophin can not be developed (Figure 2D).

Non-uniform dystrophin expression

In some lines of transgenic mice dystrophin was produced in a variable pattern, likely representing position effect variegation due to site of intégration effects [14]. In thèse lines, some fibers express very low levels of dystro-

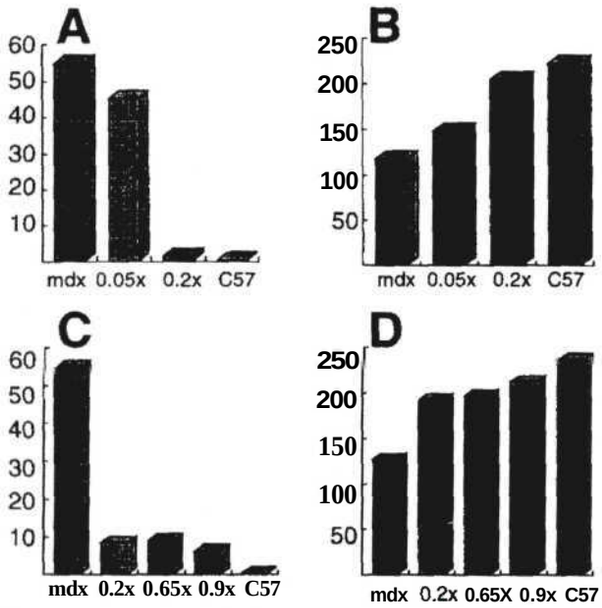

Figure 2. Degree of phenotypic correction of the mdx *mouse pathology resulting from various levels of dystrophin expression in diaphragm muscles. A and C: percentage of centrally nucleated fibers; B and D: Specific force (kN/M~). A and B: transgenic* mdx *mice expressing the full-length muscle isoform of dystrophin; C and D: transgenic* mdx *mice expressing the internally truncated dystrophin corresponding to a deletion of exons 17-48. C57 refers to wild-type C57Bl/10 mice, 0.05x refers to an animal expressing 5% of wild-type dystrophin levels.*

phin, while others express moderate to high levels [17, 18]. The uniformly expressing mice provide useful data on the overall levels of dystrophin needed to prevent dystrophy, while the non-uniformly expressing lines provide data on the percentage of fibers that must be transducer to alleviate symptoms. Comparison of the dystrophic pathology of several such lines indicated that at least 50% of the muscle fibers must accumulate moderate amounts of dystrophin to prevent a severe dystrophy [17]. For example, Figure 3 displays the muscle morphology and dystrophin immunostaining pattern a transgenic mouse line that expressed dystrophin in a variable pattern. Western analysis indicated this transgenic line expressed approximately 20% of wild-type levels of dystrophin, which should have resulted in normal muscle morphology. However, the mild dystrophy present in this mouse line illustrates that non-uniform distribution of dystrophin is not fully able to prevent dystrophy. As with the low expressing lines described above (Figure 2), many of the mosaic animals did display a milder dystrophy than do *mdx* mice. These data suggest than delivery of even small amounts of dystrophin should always lead to an improvement in muscle pathology but uniform distribution of moderate levels of dystrophin will be needed for a complete correction of this disorder. In addition, the mild phenotype observed in the mosaic lines

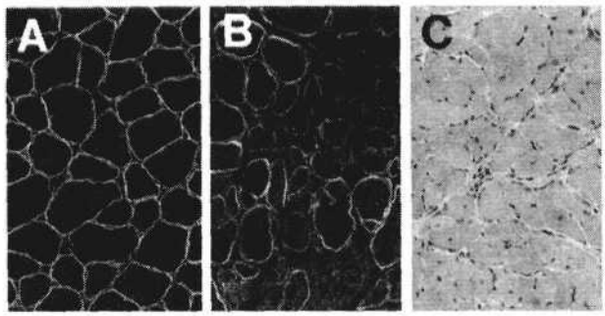

Figure 3. Morphological and immunofluorescent analysis of diaphragm muscle from a transgenic mouse with a mosaic pattern of dystrophin expression. A) dystrophin immunostaining in a wild-type mouse diaphragm; B) dystrophin immunostaining in a transgenic mouse line (line 12157-CVBA) that expressed approximately 20% of wild-type levels of dystrophin, however the dystrophin was expressed in a mosaic pattern; C) Haematoxylin and eosin stained 4 μM sections from the same mouse as in B. This distribution of dystrophin resulted in a less severe pathology than present in age matched mice, but was greater than in mice with a uniform distribution of dystrophin [17].

indicates that dystrophin positive fibers must be able to exert a protective effect on neighboring dystrophin negative fibers [17, 18].

Discussion

The transgenic mouse experiments using dystrophin expression vectors indicate that an effective treatment for DMD could be accomplished if moderate levels of dystrophin could be restored to the majority of muscle fibers in critical muscle groups. Dystrophin constitutes a minor percentage of the total protein in muscle fibers [8], and our studies indicate that approximately 20% of the wild-type levels should suffice for treatment. Experiments in mice must be interpreted with caution, but studies of dystrophin levels in female DMD carriers and mildly affected patients suggest that similar distributions and required levels of dystrophin will likely be needed in human patients [2, 9]. A slight variability in the required levels of dystrophin in limb versus diaphragm muscles has been suggested, and will need to be considered in clinical trials [17]. Although these experiments have been performed in mice, a number of our transgenic lines expressed human dystrophin cDNAs, which resulted in similar effects as the murine clones [17]. However, the enormous amount of muscle tissue present in humans suggests that restoring the required dystrophin levels in all muscles could be extremely difficult. Initial treatment approaches might be limited to targeting certain critical muscles in the body, such as the diaphragm and intercostal muscles. However, even with these groups our data suggests the majority of muscle fibers within each muscle bundle will need to be converted to a dystrophin positive phenotype.

Delivery of dystrophin to muscle via myoblast transfer or direct gene delivery has the potential to elicit an immune response against dystrophin, since most DMD patients do not express portions of this protein. However, since utrophin is not mutant in DMD patients, gene transfer of utrophin or pharmocologic upregulation of utrophin expression could be a viable alternative to dystrophin replacement.

Indeed, utrophin has been shown to substitute for dystrophin when ectopically expressed in *mdx* mouse muscles [22]. Nonetheless, additional experiments will be required to determine whether intracellular dystrophin expression in muscle is able elicit either a humoral or a cellular immune response, or even whether ectopic expression of high levels of utrophin could break tolerance and lead to rejection of tissues overexpressing utrophin.

Another potential concern in developing a treatment for DMD and the related Becker muscular dystrophy (BMD) is whether the abnormal dystrophin generally present in BMD patient muscle will interact with exogenously delivered dystrophin to create a dominant negative phenotype. However, studies involving expression of internally truncated dystrophins on a wild-type mouse background have not revealed any dominant negative interactions of the dystrophin molecules [3]. These data indicate any treatment for DMD should be applicable to BMD patients as well, and also suggest that dystrophin may not form dimers that would be subject to dominant negative interactions.

The major concern with a treatment for DMD remains how the required levels of dystrophin could be delivered to muscles. Myoblast transfer is one method, and is discussed in detail elsewhere in this journal issue. Alternate approaches include efficient gene transfer methods that might lead to transduction of muscle with dystrophin or utrophin cDNAs, including the use of viral vectors, naked DNA, or lipid-DNA complexes for gene delivery [7, 20]. Another potential approach is the use of pharmacological methods that might upregulate the endogenous utrophin gene [21, 22]. Whether any of these approaches can lead to a successful treatment for DMD or BMD will depend on whether they are able to generate sufficient dystrophin (or utrophin) in the majority of muscle fibers to eliminate disease progression.

A major unanswered question is at what age a patient can be successfully treated by any of these methods. Delivering large quantities of dystrophin to a patient with little remaining muscle tissue would not likely lead to an effective treatment, unless methods such as myoblast transfer can succeed in replacing lost muscle. The required timing for a gene transfer of upregulation protocol can be estimated from transgenic mouse studies using an inducible dystrophin gene [1]. One such line of mice using a tetracycline inducible promoter has been described, and further studies of these animals may reveal how late into the disease process a gene transfer approach might be successful.

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