Molecular Therapeutic Intervention for Dystrophin-Deficient Muscles

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June, 2000

A thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

Acknowledgment

First and for most I would like to express my profound gratitude to Dr Petrof for having welcomed me in his laboratory, for his constant support and help. He truly inspired me to challenge myself and instilled in me the critical and questioning approach to scientific research. I am forever indebted for all he has done for me.

I would also like to thank my lab-mates both past and present, Johanne Bourdon, Neola Matuseiwicz, and Naila Chugtai for their precious help and constant availability. Dr Won Kyung Cho, Dr Satoru Ebihara, Dr Lying Yang, Roy Dudley and Dr Gawiyou Danialou for their endless supply of cheer, and for engaging me so frequently in thought-provoking scientific discussions. I also acknowledge Anjte Bier, Dominique Besso and Elisa Monaco for their friendly assistance with all the administrative work.

I would like to thank Steve Prescott for supplying me with reagents whenever I was unexpectedly running out of the products and for putting aside for me the old mdx mice. I also extent these warm thanks to Carol Allen for sharing her technical expertise in immunocytochemistry techniques.

Let me express my profound gratitude to *LA DIRECTION GÉNÉRALE DES BOURSES ET STAGES* (Government of Gabon), who through le projet bourses et stages of CEGEP St Jean sur Richelieu, (particularly Ms Suzanne Boisvert) have been providing me with moral and financial support during all these years.
I could not have accomplished this work without the continuous encouragement of my family, my mother, my sisters and brothers, particularly Mibemu S. Guibinga who provided stress-release by always making me laugh.

Finally, I thank all the research, students and administrative staff at the Meakins-Christie laboratories and at the Molecular Endocrinology laboratories at the Royal

Victoria Hospital for their friendly assistance and for having provided me with the pleasant working environment which have been a second home for me.

... I've been climbing stairs but mostly stumbling down, I've been reaching high but always loosing ground, You see I've conquered hill but I still have mountains to climb...
Nevertheless I know that someone up there likes me

I dedicate this thesis to my mother **MARIE-DELPHINE BAKENDA** and to my late brother **LOUIS-GEORGES ETENO** with all my love.

Contribution of Authors

I have chosen to present the current thesis as a dissertation which takes the format of a collection of research papers. In order to inform the external examiner of the faculty regulations concerning this format of thesis preparation, I have reproduced in full form the guidelines for thesis preparation of McGill Faculty of Graduate Studies.

Candidates have the option of including as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis and reprints of published papers can included in the appendices.

The thesis must be written as a cohesive unit with a logical progression from one chapter to the next rather than as a collection of manuscripts. Connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must still conform with all other requirements of the "Guidelines for thesis preparation". The thesis must include: a table of contents, an abstract in both French and English, an introduction which clearly states the rationale and objectives of the research, a review of the literature, a final conclusion or summary and a comprehensive reference list.

Additional material must be provided, where appropriate (eg. in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of research reported in the thesis. In the case of co-authored papers, the candidate is required to make an explicit statement in the thesis as who contributed to such work and to what extent. Supervisors must attest to the accuracy of this statement at the doctoral oral defense. Since the

task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

The data presented in this thesis have been published or are to be submitted to peer-reviewed journals for publication. The following individuals appears as co-authors, in addition to Ghiabe H. Guibinga on some of these papers and their contributions are as follows:

From Dr Petrof Laboratory at the Royal Victoria Hospital and the Meakins-Christie Laboratories

Dr Ebihara, Satoru:
Former post-doctoral fellow in Dr Petrof's laboratory, he performed conjointly with me the majority of the muscle contractility experiments described in chapter 3 and 5, as well as the histological analysis described in the chapter 5.

Dr Petrof, Basil J.
The supervisor, his contribution to this thesis is intellectual, financial and moral.

From the Neuromuscular Research Group at the Montreal Neurological Hospital (MNH)

Dr Gilbert, Renald:
Post-doctoral trainee of the Neuromuscular Research Group, he was involved in the adenovirus-utrophin design and purification described in chapter 5.

Dr Karpati, George
Head of Neuromuscular Research Group at MNH. Most of the work described in this thesis involving adenoviral purification was carried out in his laboratory.

Dr Lochmuller, Hans

Former post-doctoral trainee in Dr Karpati laboratory, he helped with immunocytochemistry, and anti-dystrophin antibodies detection described in this chapter 4.

Dr Nalbantoglu, Josephine

Director, at Neuromuscular research group in MNH, she was implicated in most of the experimental works described in this thesis involving molecular biology and adenovirus vector production.

From Biotechnological Research Institute (BRI) of the National Research Council of Canada

Dr Massie, Bernard

His laboratory participated in production and construction of the recombinant adenovirus vectors described in the chapters 3, 4 and 5.

As concerns the thesis regulations on presentation of figures, I have chosen the option of presenting the figures on full unnumbered pages with figure legends on a separate page. May the reader take note that the figure legend precedes the corresponding figure.

This thesis contains the texts adaptated from the following papers

Chapter 2. Functional response of normal and dystrophic (mdx) diaphragms after notexin-induced muscle regeneration.

Ghiabe H. Guibinga, *B.J. Petrof (to be submitted to Journal of Neurological Science)*

Chapter 3. Functional consequences of adenovirus-mediated mini-dystrophin gene transfer in experimentally regenerated muscle of old dystrophic (mdx) mice.

Ghiabe H. Guibinga; *S. Ebihara; B. Massie; J. Nalbantoglu; G. Karpati; B. J. Petrof. (to be submitted to Neuromuscular Disorders)*

Chapter 4. Combinatorial blockade of calcineurin and CD28 Signaling facilitates primary and secondary therapeutic gene transfer by adenovirus vectors in dystrophic (mdx) mouse muscles.

Ghiabe H. Guibinga, *H. Lochmuller; B. Massie; J. Nalbantoglu; G. Karpati; Basil J. Petrof (Published in J of Virology, 1998, 72:4601-4609)*

Chapter 5. Overexpression of dystrophin and utrophin by adenovirus-mediated gene transfer produces differential effects on muscle function in adult immunocompetent dystrophic (mdx) mice.

Satoru Ebihara; ***G. H. Guibinga;*** *R. Gilbert; B. Massie; J. Nalbantoglu; G. Karpati; B.J. Petrof (Accepted for Publication in Physiological Genomics (2000).*

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List of Abbreviations

Achr	Acetylcholine receptor
AdV	Adenovirus Vector
APC	Antigen presenting cell
Bp	Base pairs
AdV-Dys	Adenovirus-Dystrophin
AdV-Utr	Adenovirus-Utrophin
CAR	Coxsackie Adenovirus Receptor
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
DG	Dystroglycan
DGC	Dystrophin associated protein complex
DMD	Duchenne muscular dystrophy
DNA	Dexoxyribonucleic acid
FGF	Fibroblast growth factor
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
Kda	Kilodalton
Kb	kilo-base
LFA	Leukocyte function antigen
LGMD	Limb girdle muscular dystrophy
µg	Microgram
Mdx	Murine dystrophy X-linked
MHC	Major histocompatibility complex
MHC	Myosin heavy chain
MTJ	Myotendinious junction
Nm	Nanometer

NMJ	Neuromuscular junction
OD	Optical density
PCR	Polymerase chain reaction
SG	Sarcoglycan
SPN	Sarcospan
TGF	Transforming growth factor
TCR	T cell receptor

Abstract

Duchenne muscular dystrophy (DMD) is common and fatal X-linked genetic disorder. DMD is characterized by the lack of skeletal muscle regeneration, particularly at the end stage of the disease progression. Although pharmacologic treatments can slow down the disease progression, a more definitive therapy is needed and would require replacing the missing gene product, dystrophin. Therefore, the overall objective of this thesis was to carry out a prospective study for dystrophin gene therapy in dystrophic muscles, using the X-linked muscular dystrophy (mdx) mouse model. Recombinant adenovirus (AdV) is presently the vehicle of choice for gene therapy for a number of diseases including DMD. However AdV possess two major limitations when utilized as vectors in skeletal muscles: (i) the maturation-dependency of AdV-infectivity in skeletal muscles, (ii) the host immune response against adenoviral proteins as well as the transgene product. Thus, the work presented in this thesis addresses these two major limiting factors. By modifying either the host or the vectors, we have attempted to optimize AdV-mediated therapeutic gene transfer in dystrophic muscle. Our strategy has consisted firstly to evaluate the regenerative response of dystrophic muscle with advanced disease after experimentally-induced regeneration, in an attempt to recapitulate the myogenic program. We report that mdx mice with severe dystrophic pathology can still show a substantial level of muscle repair with attendant generation of immature myofibers. Secondly, by taking advantage of this level of regeneration and the concomitant generation of immature myofibers, we have upregulated expression of the coxsackie adenovirus receptor (CAR) present in mdx muscles. CAR is the attachment receptor of AdV that is essential for an efficient AdV transduction. We have delivered AdV containing a dystrophin gene (AdV-Dys) at a period corresponding to this peak level of CAR expression and we have reported a significant increase of the number of dystrophin expressing myofibers with a net trend toward muscle function amelioration.

To address the no less important immunological issues, the work of this thesis also reports that the combined blockade of calcineurin and CD28 signaling, two key and distinct elements needed for an effective immune response, effectively blunted the immune-mediated destruction of dystrophin expressing myofibers expressed after AdV-Dys delivery. As an alternative to host modification (regeneration and immunosuppression) that can be associated with potential toxic effects, we have explored a strategy where by the recombinant AdV vector contains a less immunogenic transgene utrophin. Utrophin is highly homologous to dystrophin and present in DMD patients and mdx mice. It therefore does not constitute a neoantigen in the context of dystrophin deficiency. Based upon previous transgenic animals studies, it has been demonstrated that overexpression of utrophin in dystrophin deficient muscles can prevent the dystrophic phenotype. We report that overexpression of utrophin and dystrophin by AdV-mediated gene transfer in adult immunocompetent mdx mice produces differential effects on muscle cell function in adult immunocompetent (mdx) mice. Interestingly, we have noticed that AdV-mediated utrophin gene transfer in immunocompetent mdx mice possesses an advantageous therapeutic ability compared to AdV-mediated dystrophin gene delivery. This is evidenced (for AdV-mediated utrophin) by a greater persistence of the therapeutic transgene, the decreased inflammation and finally the improvement of muscle force generating capacity.

Taken together, it is hoped that the findings of this thesis will help in the design of gene therapy for DMD.

Résumé

La dystrophie musculaire de Duchenne (DMD) est une maladie génétique grave liée au chromosome X. Elle est caractérisée par l'absence d'une protéine, la dystrophine, dont l'absence conduit à une large destruction des fibres musculaires squelettiques et une invasion du tissu conjonctif, particulièrement dans les étapes finales de l'évolution de cette affliction. Bien que les traitements pharmacologiques permettent aujourd'hui de ralentir la progression de la DMD, une approche thérapeutique permanente est souhaitée. La thérapie génique qui consiste à remplacer le gène défectueux de la dystrophine pourrait offrir une solution définitive. Par conséquent, l'objectif de cette thèse a été d'effectuer une étude prospective sur la thérapie génique dans les muscles dystrophiques. Nous avons utilisé un modèle dystrophique murin, la souris mdx qui a la particularité de présenter le même défaut génétique que chez l'homme. Les adénovirus recombinants (AdV) sont présentement les véhicules de choix dans la thérapie génique d'un grand nombre de maladies, incluant la DMD. Les AdV présentent néanmoins deux inconvénients majeurs pour leur utilisation efficace dans les muscles squelettiques: (i) la capacité d'infection dépend de l'état de maturation de la fibre musculaire, (ii) la réponse immunitaire de l'hôte contre les antigènes viraux et le transgène. À cet effet, le travail présenté dans cette thèse s'est efforcée de surmonter ces inconvénients. Notre stratégie a consisté dans un premier temps à induire expérimentalement la régénération musculaire en utilisant la notexin, substance myonécrotique, et à évaluer la réponse regénérative du muscle dystrophique dans un état pathologique avancé. Nous rapportons que les souris mdx sont encore capables de régénéresence musculaire et par conséquent capables de produire des fibres musculaires immatures. Dans un second temps, en tirant avantage de ce niveau de régénération et de l'augmentation concomitante de myofibres immatures, nous avons caractérisé des niveaux élevés du récepteur d'attachement de l'adénovirus, CAR. L'attachement à CAR par les adénovirus est une étape essentielle à une transduction et expression effective de la cellule musculaire.

Nous avons transféré l'adénovirus contenant le gène de la dystrophine (AdV-Dys) à un moment qui correspondait à une expression marquée de CAR. Notre travail a démontré une augmentation significative du nombre de fibres musculaires exprimant la dystrophine. Parallèlement, nous avons noté une nette tendance vers l'amélioration de la fonction musculaire. Pour aborder l'aspect immunologique des inconvénients liés à l'utilisation des AdV, nous avons bloqué simultanément les voies de la calicineurine et du CD28, qui sont deux éléments clés pour une réponse immunitaire efficace, et nous avons prévenu la destruction des fibres musculaires exprimant la dystrophine suite au transfert de l'AdV. Comme approche alternative aux modifications apportées chez l'hôte (régénération et immunossupression), nous avons évalué une stratégie qui a consisté à remplacer le gène de la dystrophine par celui d'un homologue, moins immunogénique, l'utrophine. L'utrophine est exprimée aussi bien chez les patients DMD que chez la souris mdx et ne constitue donc pas un néo-antigène. Sur la base d'expériences effectuées sur les souris transgéniques, il fut démontré ultérieurement que la surexpression de l'utrophine dans les muscles déficients pour la dystrophine peut efficacement mener à une réduction du phénotype dystrophique. Nous avons démontré que la surexpression de l'utrophine et de la dystrophine dans les muscles des souris mdx adultes immunocompétentes grâce à l'AdV, produit un effet différent sur la fonction musculaire. Parrallelement, nous avons noté que le transfert de l'utrophine (par l'AdV) entraine une plus grande expression du transgène, une diminution du processus inflammatoire et finalement une amélioration de la fonction musculaire.

Dans son ensemble, il est souhaité que les résultats rapportés dans cette thèse puissent aider à l'élaboration de thérapie géniques pour les patients atteints de DMD.

CHAPTER 1.

Introduction and Review of literature

1.1 Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked genetic disorder that affects 1 in 3500 boys. It is caused by a mutation within the dystrophin gene that encodes a large cytoskeletal subsarcolemmal protein, dystrophin (86). Dystrophin provides a crucial link between the actin cytoskeleton and a group of proteins anchored in the cell membrane, the dystrophin protein complex (DPC) (59). The dystrophin-DPC axis is believed to play a pivotal role in providing structural reinforcement to the muscle cell surface membrane (27;179). Thus, it protects the myofibers against mechanical stress developed during muscle contraction (136;179). Consequently, the absence of dystrophin causes a structural weakening of muscle cell membrane that engenders muscle degeneration. Early in the disease process, the degeneration is compensated for muscle regeneration (99). Unfortunately, as the disease progresses the regeneration is less effective and a loss of myofibers along with replacement by connective and fat tissue occurs. This leads to a reduction of force generating capacity and eventual severe muscle wasting. Ultimately, the involvement of cardiac and respiratory muscle leads to the death of patients. There is thus far no effective therapy to prevent the fatal outcome in individuals afflicted with DMD. Conceptually, the most direct approach to therapeutic intervention would be to supplement the defective gene with a functional gene that would halt or slow the degenerative process.

In the present thesis, using a murine model of DMD, the dystrophic (mdx) mouse that also lacks dystrophin, we have elaborated strategies to optimize the delivery of recombinant adenovirus vectors carrying the therapeutic dystrophin gene (AdV-Dys). Thus, the adenovirus vector (AdV) has shown promise for delivering therapeutic genes to muscle. AdV possesses attractive features including an ability to infect post-mitotic cells such as skeletal muscle. Our group and others

have previously reported that AdV-mediated dystrophin gene transfer in dystrophic muscles can rescue the myofibers from damage and can alleviate loss of the force generating capacity (45;187). The reaching of a favorable outcome in mdx mice after AdV-mediated dystrophin gene transfer depends on overcoming two major obstacles: (i) the maturation-dependent loss of infectivity to adenovirus of myofibers (3;61;91); and (ii) the immune-mediated destruction of dystrophin positive myofibers found after AdV-Dys delivery. Regarding the first obstacle, AdV-Dys delivery is mostly effective in immature myofibers (5;61). Concerning the second, AdV-Dys elicits a vigorous immune response against both adenoviral proteins and the transgene product dystrophin, since the latter constitutes a neoantigen in the context of dystrophin deficiency. This is the main cause for the loss the transduced cells and the resulting ineffectiveness of AdV-mediated gene therapy in immunocompetent animals (188-191). Accordingly, the specific objective of this thesis is to optimize vector-host interaction in order to facilitate the delivery of therapeutic genes in dystrophin-deficient muscles. The optimization strategy has consisted of modifying either host factors or the vector. The modification of the host was carried out by inducing muscle regeneration and by immunosuppression, while the vector construct was modified by using of less immunogenic transgene, utrophin.

Concerning the induction of regeneration, previous experiments have documented that myofibers lose infectivity by AdV as they mature (61;91). This is mainly due to the down-regulation of the coxsackie adenovirus attachment receptor, (CAR), which is a key element needed for efficient adenoviral transduction of the cells (131). In the setting of AdV-mediated gene transfer to mature muscles, this constitutes a major problem. In DMD clear pathological diagnosis of the disease is often made at the time when most muscle fibers demonstrate maturation (184). It has been widely reported that the induction of regeneration with myonecrotic substances such as notexin can allow the skeletal muscles to recapitulate the myogenic process and generate immature myofibers

(36;82). This could potentially permit generation of newly immature myofibers that would fulfill the CAR expression requirement for AdV infectivity. Therefore, if the induction of regeneration is followed by the delivery of AdV-Dys, one could potentially increase the level of the therapeutic gene expression in diseased muscles. Accordingly, in the current thesis, we have firstly evaluated the regenerative capacity of dystrophin-deficient muscles with advanced dystrophic pathology, following the administration of notexin. The injection of a myonecrotic/myoregenerative agent such as notexin has allowed the mimicking of the skeletal muscle micro-environmental conditions that prevail in young mdx mice and that have previously permitted efficient transduction by AdV-Dys (4;6).

It is worth emphasizing that there are certain differences between mdx mice and DMD with respect to the severity of the dystrophic phenotype. In mdx mice, the limb muscle pathology is reduced compared to DMD patients. This has been attributed to a more effective regenerative capacity of mdx muscles (10;196). In contrast, the diaphragm muscle of mdx mice experiences more severe dystrophic pathology and reproduces most features developed in the human disease (156). Similarly, old dystrophic mice harbor more severe limb muscle pathology, such as a substantial decrease of the regenerative capacity of the muscle along with proliferation of connective tissue(114). Therefore, the mdx mouse diaphragm and limb musculature from old mdx mice appear to constitute from a biological standpoint an acceptable tool to evaluate the efficacy of AdV-mediated dystrophin gene transfer in dystrophic muscle with advanced pathology.

With regard to the immune-mediated destruction of dystrophin expressing myofibers after AdV-Dys delivery, in presence of an intact immune system, CD8+T cells destroy the AdV-infected myofiber population (58;188-191). However, the administration of immunosuppressive regimens has permitted one to limit the immune response associated with AdV-based gene therapy and allow a more sustained expression of the transgene (96;188;189). The employment of

conventional immunosuppressive agents such as FK506, which acts by interfering with CD3 T cell receptor-mediated lymphocyte activation, is efficient for maintaining the level of dystrophin expressing myofibers after AdV-Dys delivery (118). However, it is only partially effective for blocking the generation of neutralizing antibodies against adenoviral capsid proteins, known to prevent efficacious vector re-administration (118). Since T cell activation could also occur via redundant signaling pathways that are unaffected by the blockade of TCR signaling events (68;97), we have hypothesized that targeting of multiple key steps required for effective immune response stimulation would offer a better protection against the host immune response that mediates destruction of AdV-transduced myofibers after AdV-Dys delivery.

It is now well documented that the transgene product as well as adenoviral proteins can be a major target for the immune-mediated destruction of myofibers after AdV delivery (164). Thus, for dystrophin itself, which in a context of dystrophin-deficient species is immunogenic (118;132), it would be of particular interest to utilize a transgene that is therapeutically equivalent to dystrophin but which does not elicit an immune response. Utrophin, formerly known as dystrophin-related protein, has emerged as a potential candidate to replace dystrophin in the context of gene therapy for dystrophin deficient muscles. In vitro studies have for instance shown that utrophin is capable of binding both to actin and to the DPC (25). Furthermore, it has been reported that in normal fetal muscle, utrophin is localized to the sarcolemma before being replaced gradually by increasing levels of dystrophin during the development (31;106). Based upon previous transgenic animal studies, it has been demonstrated that overexpression of utrophin in dystrophin deficient muscles can prevent the dystrophic phenotype in mdx mice (161).

Specifically, the present thesis covers six chapters. Chapter 1 is the review of literature that deals with the molecular biology of the dystrophin gene as well as

the cellular and molecular basis of skeletal muscle injury in relation with dystrophin deficiency. It also discusses the usefulness of adenovirus-mediated gene transfer in muscle and the overall problems associated with this therapeutic approach. Chapter 2 initiates the study of maturation-dependence of AdV infectivity in severely affected dystrophic muscles. It addresses the regenerative capacity of dystrophic muscle with severe pathology after experimental regeneration induced by notexin. We demonstrate that the use of notexin in advanced disease does not lead to major functional deleterious effects. In expanding upon these findings in Chapter 3, we have forced the generation of immature myofibers in muscle with advanced dystrophic pathology that normally regenerate poorly and subsequently, we have delivered AdV-Dys in muscles at the time of peaked CAR expression. The histological and functional evaluation of the risk: benefit ratio associated with the treatment is addressed. Chapter 4 deals with immunological problems associated with the loss of transduced myofibers after AdV-Dys delivery. We demonstrate that by interfering conjointly with calcineurin and CD28 signaling, two mandatory events for activation and co-stimulation of the immune response, one can facilitate primary and secondary therapeutic gene transfer by adenovirus vectors in dystrophic mouse muscles. In Chapter 5, we have modified the transgene and therefore, we have approached the immunological paradigm differently. The chapter discusses the possibility that utrophin, because of its intrinsic lower immunogenicity compared to dystrophin in dystrophin-deficient animals, could produce different functional and histopathological effects comparatively to dystrophin, when provided in the context of AdV-mediated gene transfer in adult immunocompetent dystrophic mice. Finally, Chapter 6 concludes the thesis, and discusses the perspectives and contributions of the findings of the thesis to the existing body of scientific knowledge.

1.2 Early beginnings in muscular dystrophy: Historical perspective

Duchenne muscular dystrophy was named after the French physician *Guillaume Benjamin Amand Duchenne de Boulogne. Duchenne* main research interest was the electrical stimulation of muscle, muscle function and neuromuscular disorders. In his book "électrisation localisée" Duchenne described in detail the first clinical and histological features of what appears to be the manifestations of DMD. Duchenne designed a needle-harpoon (emporte-piece histologique) to collect biopsy specimens. This technique allowed him to study material from the same patient at different stages of the disease and therefore confirm the progressive nature of the disease (55). Thus, before this major technical achievement, the pathological diagnosis was performed at autopsy. Duchenne further defined pathological traits such as the "paralysie myosclerique" or myosclerosis paralysia that is so often noticed in dystrophic muscle (55). The later clinico-pathological feature is characterized by widespread invasion of connective tissue in muscle, which led Duchenne to use the terminology hyperplasia of connective tissue. By clarifying the pathology of this disease, Duchenne also dismissed the conception of the moment that the disease may be caused by a lesion of the spinal cord as *Dr Edward Meryon* (1809-1889) had previously put forward (55).

Another important contributor to the understanding of myopathy is *William R. Gowers* (1845-1915). Aside from reaffirming the observations already made by *Duchenne*, like noticing the Pseudo-hypertrophic muscular paralysis so indicative of the affliction, *Gowers* succeeded in giving a more complete picture of disease in term of describing the clinical features. He pointed out what is nowadays known and referred to *as Gower's manoeuvre or Gowers' sign*. In other words, he made the observation that weakness of the hip and knee extensor leads to a difficulty in rising from the floor or a chair. In addition to these descriptions, *Gowers* recognized the hereditary nature of the disorder and drew a correlation

with haemophilia (another X-linked genetic disorder), by noticing the predilection for males. Based upon the clinical and pathological observations of Gowers and others, it was already suspected that there could be more than one disease related to the described presumed diagnosis. *Wilhelm Heinrich Erb*, (1840-1921) elaborated the possibility of the existence of a heterogeneous group of disorders within all the clinical and pathological portrayals that had been made. *Erb* circumscribed what is known today as the major pathological hallmark of the disease, muscle degeneration. He came out with the term *Dystrophia muscularis progressiva* for progressive muscular dystrophy (55).

The 20th century has seen our understanding of muscular dystrophies increased substantially, thanks to the work of pioneers of modern human genetics such Alan *E. H. Emery and Peter E. Becker*. These two and others contributed importantly to the classification of muscular dystrophy. The classification is often based on the mode of inheritance (X-linked versus autosomal recessive or dominant), the type of muscle afflicted and the onset time of disease (adult form versus childhood form). Given the wide differences that may exist between the disease phenotype among muscular dystrophies, it was then useful to standardly specify what could be categorized as muscular dystrophies:

This is a group of inherited disorders which are characterized by a progressive muscle wasting and weakness, in which the muscle histology has certain distinctive features (muscle fibre necrosis, phagocytosis, etc) and where there is no clinical or laboratory evidence of central or peripheral nervous system involvement or myotonia (Emery, 1987, (73)).

With the avenue of molecular genetics, it was now possible to precisely map the gene responsible for these diverse groups of diseases. Indeed, the cloning of the gene responsible for DMD by the group of *Louis M. Kunkel* at Harvard is one of the major accomplishments of the last 20 years in molecular genetics. Dystrophin

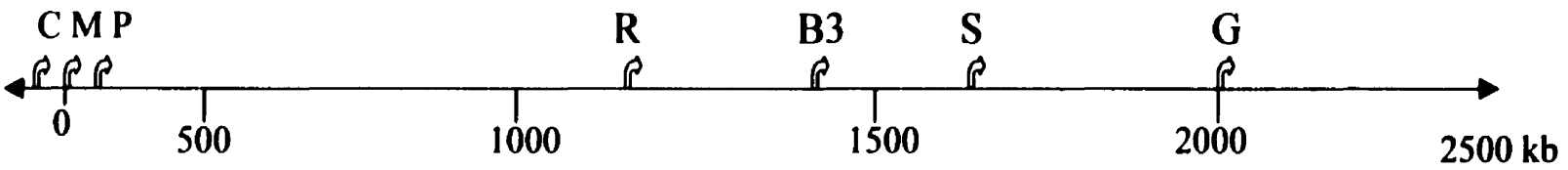
was one of the few genes discovered using the reverse molecular approach called positional cloning, i.e. identification of the gene and then identification of the protein (86).

1.3 Dystrophin gene and protein: The general overview

1.3.1 Organization of dystrophin gene

The dystrophin gene is located on the short arm of the X chromosome and is about 2.4 megabase pairs long, that consists of 79 exons (7;12;87;108). It is the largest known human gene to date, which may explain its propensity to mutation which has been estimated to be of the order of 1 in 10000 (100). With regard to the type of mutation in DMD, approximmatively 70% of DMD mutations are of micro deletion and only a small proportion (<10%) of DMD patients have duplications or point mutations (56). The central region of gene (exon 9-63), particularly the proximal portion (first 20 exons) are more prone to mutation (15). It is however noteworthy that the position and the size of mutation are independent of the clinical phenotype (9). Generally, *out of frame* deletions are associated with DMD where there is a complete absence of dystrophin. On the contrary, *in frame* deletions generated a milder phenotype, Becker muscular dystrophy (BMD) where a truncated instead of a full-length dystrophin is generated (9). Concerning the gene organization, dystrophin locus possesses 7 promoters generating 3 major full-length transcripts and 4 internal "short-length" transcripts (see figure 1). The 3 major promoters are mainly active in the cortex (promoter-C), muscle (promoter-M), and Purkinje cells (promoter-P). The full-length transcripts generated from these promoters possess a distinct first exon, spliced to share a set of 78 exons (195). A 14 kb transcript is generated from these 3 promoters which gives a protein of approximately 427 kilodaltons (kD). Beside these 3 major promoters, the dystrophin gene possesses 4 internal promoters that are active in the retina (R), brain-3 (B3), Schwan cells (S)

Figure 1. The dystrophin gene. Top line at least 7 distinct promoters drive the cell-type specific expression of dystrophin. The C (cortical), M (muscle) and P (purkinje cell) dystrophins generate the full length forms. In addition, there are 4 internal promoters that are active in the retina (R), brain-3 (B3), Schwan cell (S) and general organs (G). These promoters encode C-terminal proteins (see text). Middle line, a map of the different exon locations where the above-described promoters arise. The bottom line, schematic representation of dystrophin protein domains. [Adapted from (23)].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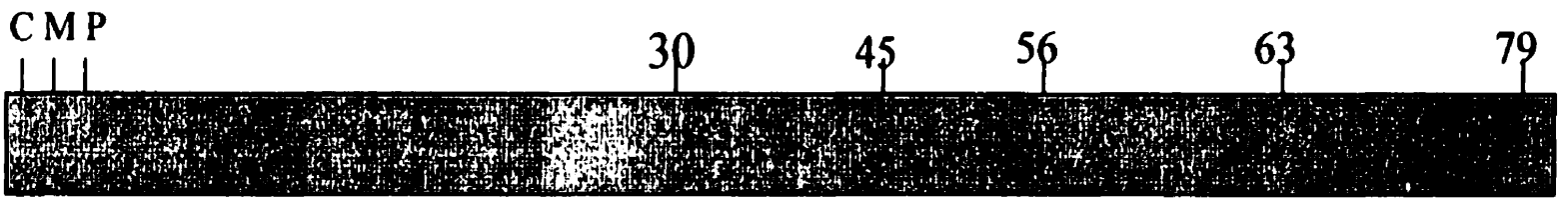
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63
79

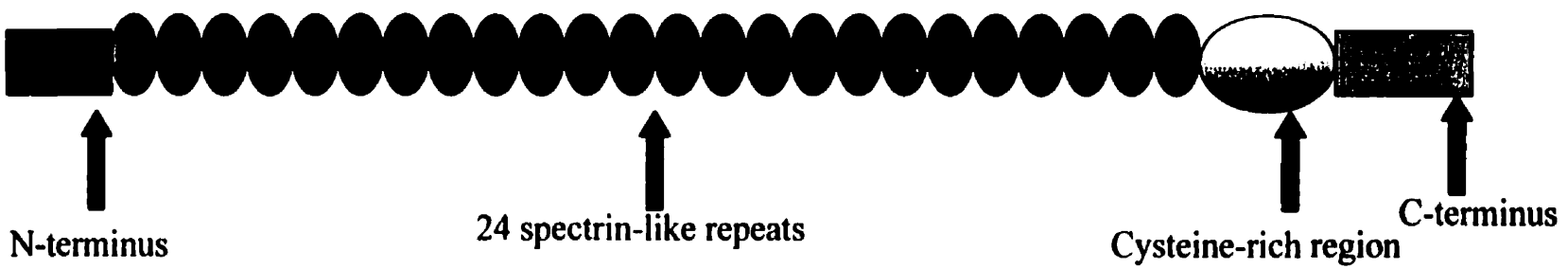

N-terminus
24 spectrin-like repeats
Cysteine-rich region
C-terminus

Table 1: Summary of the distribution of dystrophin, +, dystrophin is present; - absent: nd, not determined. OPL, outer plexiform layer.

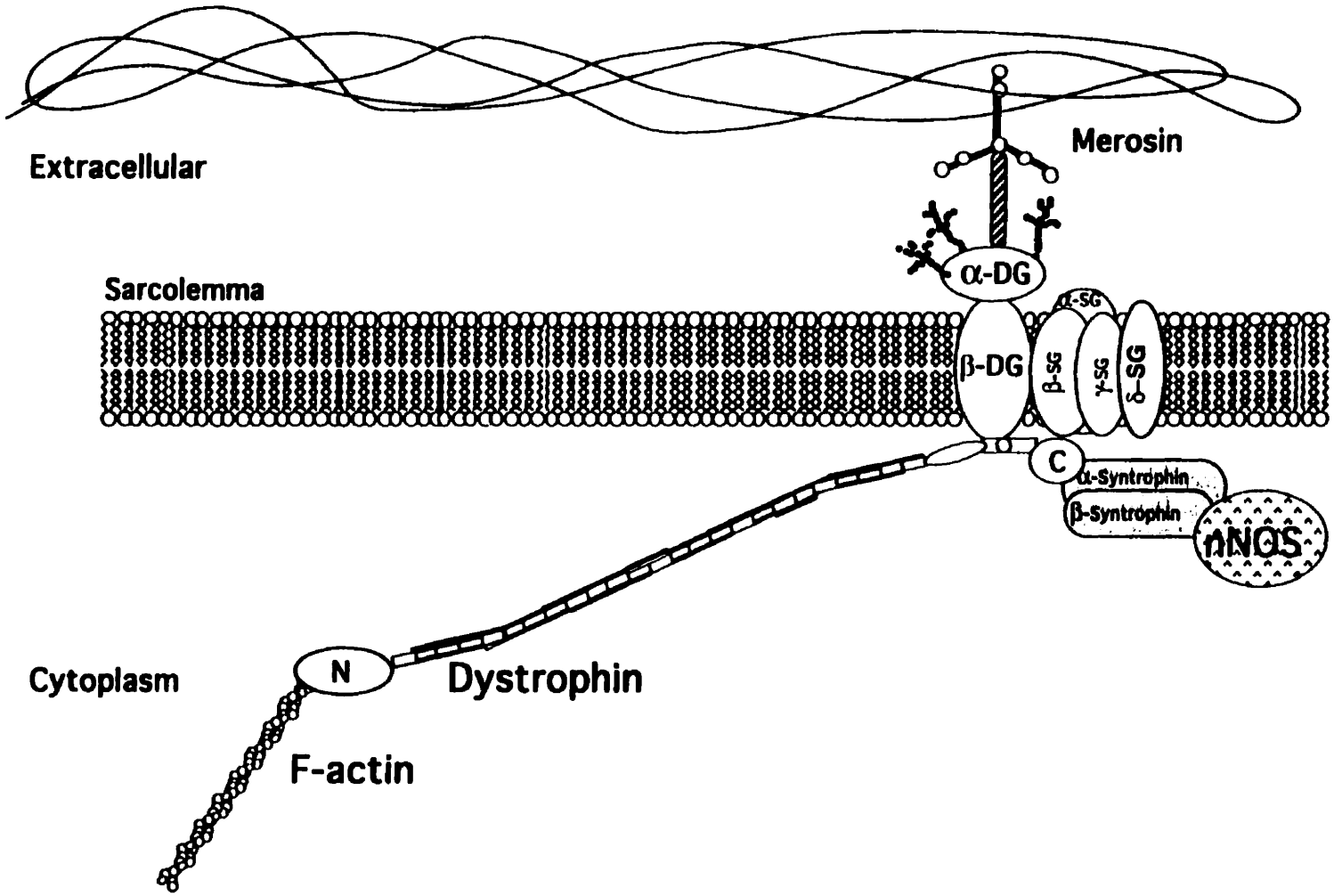
Mutually exclusive expression of dystrophin forms	427 kD	Dp260	Dp140	Dp116	Dp71
Muscle					
skeletal	+	-	-	-	-
cardiac	+	-	-	-	-
smooth	+	-	-	-	-
Organs					
kidney	-	-	-	-	+
liver	-	-	-	-	+
lung	-	-	-	-	+
pancreas	-	-	-	-	+
testis	-	-	-	-	+
Central nervous system					
neurons	+	-	-	-	nd
glia	+	-	+	-	+
Peripheral nervous system					
retinal OPL	+	+	-	-	-
neurons	+	-	-	-	nd
Schwann cell	-	-	-	+	-

and general organs (G). They encode proteins of 260, 140, 116 and 71 kilodaltons, named Dp260, Dp140, Dp116 and Dp71 respectively (see table 1). The transcript encoding the Dp71 is expressed in all organs but skeletal muscle (195) (9) (see table 1).

1.3.2 Organization of dystrophin protein domains

The full-length transcript that arises from the muscle promoter generates a protein of 427 kilodaltons (23) that has 4 domains (see figure 1). The amino terminus portion of the protein contains an actin-binding domain encoded by exons 1-8 (108). A rod-like domain consisting of 24 spectrin-like repeats and encoded by exons 9-63 constitutes the largest portion of the protein (108). Preceding the C-terminus domain is a cysteine-rich domain encoded by exons 64-67 (7). Finally, the C-terminus region is encoded by exons 68-79. Regarding the amino-terminus domain of the protein, it displays homology with α-actinin and β-spectrin, and it is through that region that dystrophin interacts with the cytoskeleton (60;85). The rod repeats fold into triple-helical coiled coils, each being separated by a hinge whose major function is to confer flexibility to the protein in response to muscle contraction for instance (98). It is however noteworthy that an actin binding site has also been identified near the middle of the dystrophin rod (147), suggesting a lateral association between dystrophin and actin (147). The cysteine rich region constitutes the third domain of the dystrophin protein. It interacts with a member of the DPC, β-dystroglycan, (see later). The C-terminus is the final domain, which interacts with other members of DPC. (See figure 2).

Figure 2. Membrane organization of the dystrophin-associated protein complex (DPC). Dystrophin conjointly with the DPC complex forms the dystrophin-DPC axis, which allows linkage of the internal cytoskeleton to the extracellular matrix (see text) [Adapted from [(24)].


Extracellular
Merosin
α-DG
Sarcolemma
α-SG
β-DG
β-SG
γ-SG
δ-SG
C
α-Syntrophin
β-Syntrophin
nNOS
Cytoplasm
N
Dystrophin
F-actin

1.3.3 Dystrophin-associated glycoprotein complex (DPC)

The DPC consists of at least ten proteins (see figure 2). β-dystroglycan interacts with the cysteine-rich region of dystrophin (95). The latter attaches to α-dystroglycan which is the receptor for skeletal muscle specific laminin-2 (merosin) in the extracellular matrix. Four distinct single-pass transmembrane proteins form the sarcoglycan complex (SG, α, β, γ, δ) (24) (22) (116). A defect in any single gene encoding one member of the SG complex can result in the loss of the entire complex (24). Consequently, this loss destabilizes the DGs and leads to a group of autosomal recessive muscular dystrophies (Limb-girdle muscular dystrophy, LGMD) which are phenotypically very similar to DMD (24) (22) (116). Another component of the DPC, the syntrophins, were originally identified as proteins enriched at the post-synaptic ends in the *torpedo electric.* The syntrophins and dystrobrevin localize at the inner surface of the cell membrane (67;157) where they bind near the C-terminus portion of dystrophin. Recently, a novel member of DPC has been identified and named sarcospan (SPN) for its four sarcolemmal spannings (tetraspan) (34;35). It has been shown to interact with the SGs to form the SG-SPN protein complex that might facilitate the interaction between all the members of DGC (34).

1.3.4 Localization of dystrophin

The identification of the gene encoding for dystrophin and the revelation of dystrophin protein domains has provided key insights for a deeper exploration of the distribution of the molecule in muscle and other organs where it is expressed. Microscopy studies involving light, confocal and electron technologies have identified dystrophin protein as being localized beneath the plasma membrane of muscle fibers (23). High resolution light microscopy studies have revealed that dystrophin is organized in a costameric fashion, co-localized with α-actinin, vinculin and talin at the level of the I-bands and Z-bands (120). On the other hand, using antibody against the large rod-domain of dystrophin, immunoelectron microscopy has revealed a micro-periodic distribution of the

molecule that appears separate from the underlying myofibrillar apparatus (38;39;174). In order to reconcile these two observations, it has been put forward that two populations of dystrophin may exist within muscle cells. The first population is mostly associated with the contractile apparatus and the plasma membrane at the I- and Z-bands. The second one as revealed by the electron microscopy studies, identifies dystrophin as a continuous sheet of molecules associated with dystroglycan and sarcoglycan complexes. The existence of these two populations has also been suggested by purification studies in which one type of dystrophin co-purified with the plasma membrane (176). The first population, mostly detergent insoluble (the major portion), remained associated with vinculin and talin (176). Beside the above-mentioned site of dystrophin location in myofibers, intense staining of dystrophin has been detected around myotendinous junctions (MTJs) (23;148;152;176). MTJs display structural specialization for force transduction from the contractile and the cytoskeletal apparatus to the extracellular matrix. The presence of extensive invaginations with finger-liked projections that increase the surface area of the end region of the myofibers has been observed (176). These structures permit a reduction of force load born per unit area of sarcolemma during muscle contraction (176). Similar structures have also been noticed around the neuromuscular junction (NMJ), where dystrophin along with agrin and the dystrophin-related protein utrophin may help to organize the acetylcholine receptor (AchR) around the NMJ (23;129;176).

1.4 Cellular and molecular basis of muscle injury and its relationship with dystrophin deficiency

1.4.1 Cell membrane integrity

Long before the discoveries leading to the identification of the gene responsible for DMD, it was speculated that the cause of DMD lay in a defect of the plasma membrane (52;54). The presence of discontinuities (called "delta lesions") could be seen in the sarcolemma of patients with DMD (26). Similar muscle cell membrane alterations have also been reported in the mdx mouse. Isolated myofibers of the mdx mouse are more susceptible than normal myofibers to osmotic shock when exposed to hypotonic solution (127). Additionally, experiments performed on myotubes have demonstrated a substantial diminution of stiffness in dystrophin deficient membranes comparatively to control (133). From a structural comparison standpoint, it is noteworthy that spectrin shares structural homology with dystrophin, and hereditary absence of spectrin in red blood cells leads to erythrocyte membrane instability with increased mechanical rupture (28;177).

Dystrophin by its direct connection to the cytoskeletal apparatus and its indirect link to the extracellular matrix mediates the dystrophin-DPC axis (see figure 2). It is speculated that the axis acts as a protective reinforcement of the muscle plasma membrane against stress generated during muscle contraction (136;179). Several experiments involving mdx mice and canine X-linked muscular dystrophy skeletal muscle support the aforementioned speculation (125;136;179). For example, mdx limb muscles subjected to eccentric contractions (contraction where the muscle is simultaneously lengthened, thus generating a high level of mechanical stress) demonstrated a higher number of IgG positive myofibers compared to normal myofibers (IgG is an index of muscle acute muscle necrosis) (179). Experiments in isolated portions of mdx diaphragm submitted to eccentric contractions showed a similar increase of damaged myofibers as determined by the uptake of Procion orange dye, which crosses the

membrane of injured myofibers but remains excluded from the uninjured cell (136). Taken altogether, these data from mdx mouse corroborate the suggestions made for DMD, that in vivo, the muscles subjected to eccentric contractions (pelvic-girdle and proximal leg muscles) during physiological activity seem to suffer particularly early and severe damage (1).

1.4.2 Calcium homeostasis

It has been reported that dystrophin deficient muscles exhibit an increased level of intracellular calcium as a direct consequence of the loss of muscle cell membrane integrity (130). A two-fold increase in total calcium content has been reported in muscle biopsies from DMD patients and from adult mdx mice (124;141;166;168). These changes in total calcium could be a secondary consequence of muscle degeneration (141). Thus, it was reported that normal calcium content was present in pre-necrotic myofibers of mdx mice, and the acute increase occurred only during the necrotic phase, with a return toward normal values after muscle regeneration (141). One the other hand, it has also been reported that dystrophin-deficient myotubes possess abnormally high permeability to calcium due to altered calcium channel activity (64;66) Accordingly, it has been hypothesized that one role of dystrophin in normal muscle is to stabilize these calcium channels, and that absence may result in the deregulation of that channel.

One possible consequence of the loss of intracellular calcium homeostasis is the activation of autoproteolytic activities. There is evidence suggesting that calpains, which are ubiquitous calcium-dependent cysteine proteases, play a role in necrosis of dystrophic muscles (153;154;167). Interestingly, Z-band proteins that constitute the structural boundary of sarcomeres (functional unit of a myofibril) demonstrate a particular susceptibility to calpains. Thus, myofibrils treated with purified calpains disorganize Z-discs and release α-actinin (21;70). A similar type of disorganization has been commonly reported as an early

pathological feature in DMD and mdx mice (153;154), where disruption of myofibrillar apparatus often precedes the appearance of necrotic events (37).

1.4.3 Cell signaling

The absence of the dystrophin protein in DMD leads to a complete absence of β-dystroglycan. Meanwhile, phosphotyrosine consensus sequences and several proline-rich regions that could associate with Src homology 2 and 3 (SH2 and SH3) domains of cytoskeletal or signaling proteins have been identified along the β-dystroglycan protein (95). Grb2, a protein that possesses SH2 and SH3 domains, has been shown to bind β –dystroglycan (95;186). It is hypothesized that β-dystroglycan in association with Grb2 might regulate cellular functions such as cytoskeletal rearrangement (186). This hypothesis is based upon previous knowledge that Grb2 modulates the activity of small GTP-binding proteins (Ras, Rac, and Rho) that stimulate cytoskeletal reorganization (122;142;143). Accordingly, the absence β-dystroglycan could deprive dystrophin deficient muscle cells of important physiological mechanisms normally involved in cytoskeletal organisation and rearrangement.

It has been demonstrated that the extracellular matrix affects a vast variety of cellular functions including cell adhesion, migration, proliferation, differentiation, attachment and viability (92;93). It has been speculated that dystrophin and other members of the DPC may play a role in extracellular matrix-mediated signal transduction (186). Indeed, association of Grb2 with β-dystroglycan once again seems to lend support to this argument. It has been reported that, in NIH3T3 cells, integrin-mediated signal transduction is coupled to the activation of a pathway involving Grb2 (149). It is speculated that the DPC complex may function in a similar manner, such that the extracellular matrix or agrin, released at the NMJ, could trigger a Grb2-mediated signaling event (186). The disruption of this interaction as observed in dystrophin-deficiency may affect normal muscle cell function and lead to muscle necrosis or apoptosis (146).

The DPC has also been implicated in cell signaling events in the L6 skeletal muscle cell line, where α and γ sarcoglycan along with the integrin system (α1β5) play functional roles in the focal adhesion complex (194), the major site of attachment to cytoskeletal actin. The focal adhesion complex becomes activated through the phosphorylation of α and γ sarcoglycan by a kinase termed focal adhesion kinase (194). In the context of dystrophin deficiency, where the sarcoglycans are reduced or absent, focal adhesion could be perturbed, affecting muscle cell adhesion and viability. Accordingly, evidence from in vitro studies with rat L6 skeletal muscle cell line suggests that the disappearance of α and γ sarcoglycan leads to a reduction of cellular adhesion (194).

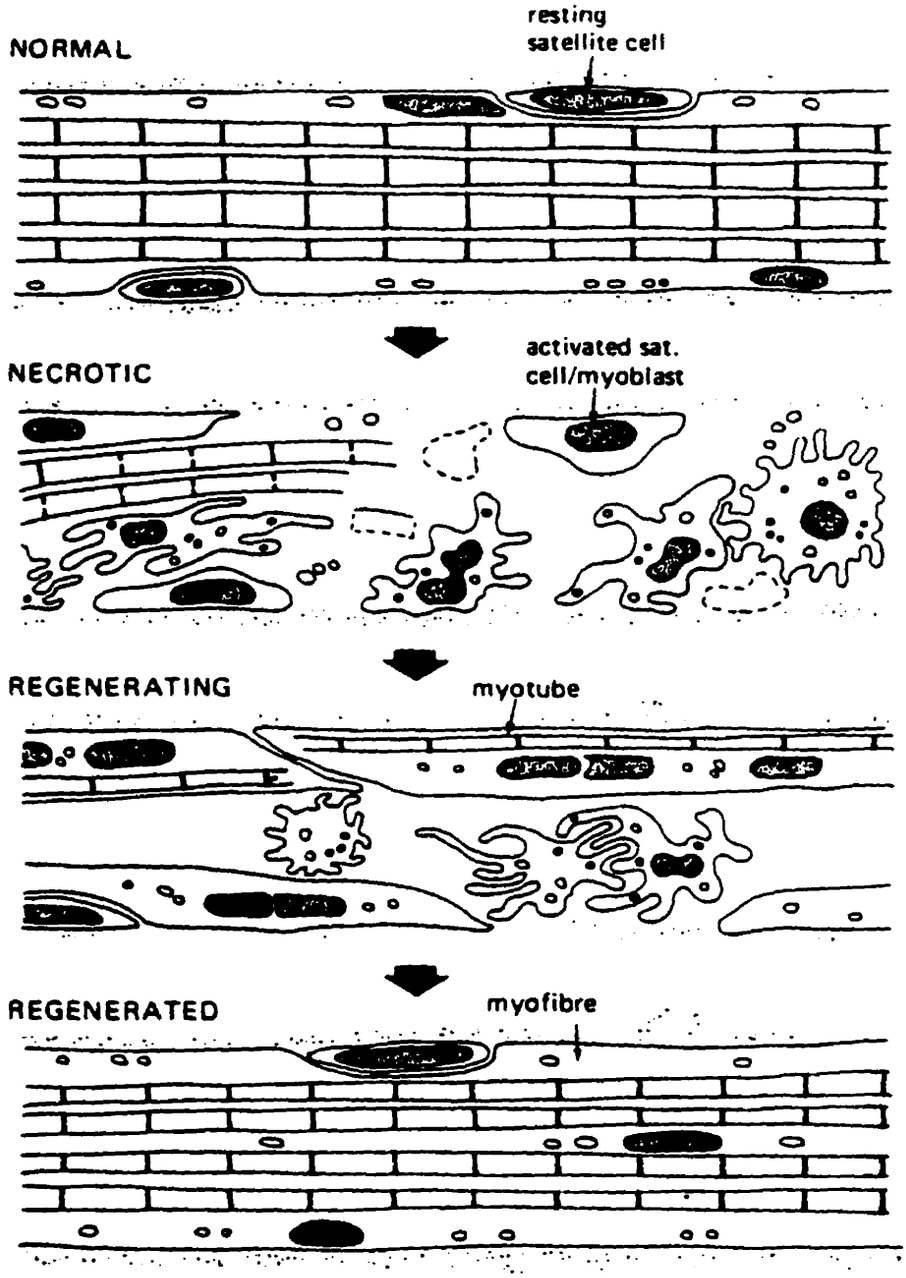
Neuronal nitric oxide synthase (nNOS), which is normally complexed with dystrophin at the skeletal muscle plasma membrane, is displaced to the cytosol in DMD patients and mdx mice, leading to a reduction in enzyme activity (22). A wide array of evidence implicates nitric oxide in various signal transduction pathways, such as its role as a second messenger in cGMP signaling pathway (47). Thus it can be speculated that the aberrant functioning of nNOS in DMD and the MDX mouse model could alter cellular signaling events, perhaps specifically at the sarcolemma, and in fact play a role in the etiology of these pathologies.

1.4.4 Necrosis and inflammation

The extensive presence of foci of necrotic fibers has been well characterized in DMD and in animal models. The definitive cause of the necrotic process has not been fully elucidated. It has been nevertheless reported that inflammation is common to both DMD and mdx mouse muscles (14;163). The augmentation of inflammatory cells such as macrophages, T cells, (mostly CD8+) and mast cells has been reported in dystrophin deficient muscles (13;14;57;126) (73). Regarding the role of macrophages, cross-breeding studies of macrophage-

deficient animals *(op/op)* and mdx did not eliminate the foci of necrosis in the double mutant (72). A role for T cells is suggested by the presence of activated cytotoxic T lymphocytes (CTLs) in DMD (13). The presence of T cell receptor (TCR) gene rearrangements at the site of muscle degeneration has also been reported as lending further support to a role for CD8+ T cells in DMD (79). In addition, it has been reported that CD8+ T cells promote apoptosis and necrosis in mdx mouse muscles (155). Thus, depletion of CD8+ T cells in mdx mice produced a significant reduction of apoptotic myonuclei along with a reduction of necrosis (155). With regard to the role of mast cells, their presence in association with progression of dystrophic pathology has been previously described (73;74). Dystrophin deficient mdx mouse muscles show mast cell accumulations that are three-fold higher than normal (74). Additionally, experiments have documented that the injection of purified mast cell granules into the gastrocnemius muscle of mdx mouse induced widespread myofiber necrosis (74). Mast cells degranulation and release of mediators such proteases and cytotoxic inflammatory cytokines could thus exacerbate the inflammation and necrosis process (72). However, it has to be pointed out that cross-breeding experiments of mdx mice with *W/W* mast cell-deficient mice have indicated that histopathology of the double mutant *mdx-W/W* is not different from wild-type mdx animals (72). However, it is possible that compensatory mechanisms from other inflammatory cell types may have taken over for the role of mast cells in this case.

Figure 3. Diagrammatic representation of regenerative events following injury in skeletal muscle. In a normal muscle, resting satellite cells are located beneath the basal lamina. Following a necrotic event, satellite cells become activated as well as inflammatory cells that initiate the phagocytic phase of the skeletal muscle repair process. The phagocytic phase is characterized by the removal of cellular debris which is mostly carried out by macrophages. After the cleansing, the regenerative phase can proceed, where activated myoblasts will fuse and give rise to myotubes. The myotubes will then reconstitute a newly regenerated myofiber (Figure taken from (56).


NORMAL
resting
satellite cell
NECROTIC
activated sat.
cell/myoblast
REGENERATING
myotube
REGENERATED
myofibre

1.4.5 Muscle regeneration

Injury to muscle that occurs either experimentally or as part of a disease process often leads to necrosis. The necrosis is normally followed by muscle regeneration (see figure 3). The regenerative process is carried out by mononucleated satellite cells (also called myogenic precursor cells, mpc) located between the plasma membrane and the basal lamina (123). Upon injury satellite cells become activated and differentiate into myoblasts that can fuse to form multinucleated myotubes that will be able to reintegrate and repair the injured myofibers (see figure 3). Recently, it has also been reported that myogenic precursor cells originating from the bone marrow play a role in the regenerative response to experimentally-induced muscle injury (63).

In DMD, the regenerative process appears to be defective, since there is replacement of myofibers by connective and fatty tissue. The question of whether the inefficient muscle regeneration in DMD lies in a reduction (or depletion) of myogenic precursors cells has been investigated. Several studies have reported that the number of satellite cells in DMD is higher than in normal muscles (40;41;175). However, Webster and Blau suggested the lack of regenerative capacity in DMD could be the consequence of a reduction of the replicative lifespan of mpc (178). Thus, their study demonstrated based upon *in vitro* experiments that the average myoblast from a 5 years old control was capable of 56 replications with a yield of about 10 cells per cell (178). However, at 2 years of age (age of the clinical disease onset in DMD), only 6% of myoblasts cultured from DMD biopsies were capable of 50 replications (178). By age 7, DMD myoblasts that were capable of 10 replications were rare (178). These findings were interpreted as suggesting that satellite cells of young DMD patients undergo repeated cycles of division in order to regenerate lost myofibers, thereby leading to premature replication senescence of the satellite cell pool.

It should be noted that some important phenotypic differences exist between DMD and mdx mouse muscles. Whilst muscle regeneration appears limited for the former as discussed above, it is quite successful for the mdx mouse (at least in limb muscle). Thus, the mdx murine model exhibits extensive necrosis at an early age (3 to 4 weeks postnatal) but the necrosis is compensated for by a complete replacement with regenerated fibers within 10 weeks (10;11). These regenerated fibers harboring central nucleation are less prone to undergo further episodes of necrosis for unclear reasons (179). It has to be pointed out, however, that the diaphragm of adult mdx mice exhibits a pattern of degeneration and fibrosis that is quite similar to that found in most DMD muscles. This includes an important level of fibrosis and a decline of force production (156). It has been proposed that the higher workload in diaphragm relative to limb muscles of mdx mice could be responsible for these differences (156).

1.4.6 Fibrosis

The failure of dystrophic muscles to regenerate is accompanied by replacement of myofibers by connective tissue. It is predicted that fibrosis in muscle could impact on muscle regeneration, since it has been noticed that regenerating myofibers can be enclosed by bundles of collagen fibrils (36). Furthermore, satellite cells that have the ability to move between and across myofibers (upon injury) could see their motility decreased by the presence of widespread fibrosis (36). The excessive fibrosis could also aggravate dystrophic pathology by decreasing vascular perfusion of the muscle (117). It could also alter the innervation of the myofibers by making nerves end abruptly on connective tissue (32). Although the causes of the intense development of fibrosis that occurs in dystrophin-deficient muscles remain partially unclear, high expression of fibrogenic cytokines such as transforming growth factor (TGF)-β1 and basic fibroblast growth factor (b-FGF) has been identified in muscle of patients with

DMD (43;185). Additionally, the level of expression of these cytokines has been correlated with muscle fibrosis in animal models of DMD (94). The deregulation of fibrogenic cytokines could be the consequence of the over-activation of wound repair mechanisms that follows the micro-environmental changes associated with muscle necrosis (74). It worth emphasizing that mast cells may play a role in the fibrotic proliferation in dystrophin deficient muscles (74). This was firstly suggested by indirect correlation with a number of disease conditions where fibrosis is prominent and where an alteration in mast cell number and activation was documented (145). In addition, mast cell components such as tryptase are potent mitogens for fibroblasts (74).

In summary, it is clear that muscle necrosis and the failure of regeneration along with connective tissue invasion provoke a severe loss of muscle fibers that is accompanied by a dramatic decrease in muscle force-generating capacity. All of the above-mentioned clinico-pathologic traits in DMD have ultimately a fatal outcome that needs to be addressed with development of any therapeutic strategy.

1.5 Therapy for Duchenne Muscular Dystrophy

1.5.1 Steroid treatment

Steroid treatment has been applied in DMD patients with a variable outcome. The anabolic steroid, 1-methyl androstenolone, has been given to several patients with muscular dystrophy including Duchenne, and 90% were reported as showing some improvement or at least no further deterioration (51). However, follow-up studies have failed to confirm its efficacy (184). Prednisone along with other corticosteroids have shown more efficacy in improving the condition of DMD patients (49;62;76). Although muscle strength and pulmonary function with the utilization of prednisone have been reported, the benefit appears to be short-lived (20;49;62;76). The prednisone may act by decreasing the invasion of CD8+ CTLs in DMD muscle (107). It is also worth emphasizing

that steroid therapy possesses numerous side effects (184). It is thus in the long run more advantageous to develop therapeutic interventions where the defective dystrophin gene could be replaced or supplemented with a normal functioning version of the gene.

1.5.2 Cell therapy

Myoblasts can be isolated from muscles and expanded in vitro. Mdx mice have been a useful model to evaluate the feasibility of this therapy with an eventual application to DMD patients. Implantation of non-dystrophic myoblasts into young mdx muscle has led to the formation of a mosaic pattern within treated muscles, which contain both donor (normal) and native (dystrophic) myonuclei (101;103). This strategy is called heterologous myoblast transplantation (HMT), and in order to maximize its efficiency has required that the host muscles be pre-irradiated to inactivate native satellite cells (101). Induction of muscle regeneration in the host is also required to facilitate the integration of donor cells (101). Because donor cells are prone to immune rejection, and it is also necessary to immunosuppress the host (101). As an alternative to HMT and the inherent immune response associated with it, autologous myoblast transplantation (AMT) has been performed in mdx mice. The approach consists of establishing mdx myoblasts in vitro, and transfecting the cultured myoblasts with a functional dystrophin cDNA. The transfected myoblasts are then reintroduced into the original host donor as described above for HMT. Clinical trials initiated to test the safety of myoblast transplantation into DMD patients have reported no deleterious effects from the injected cells (65;113). The recurrent problem is that few injected muscle cells gain access to host myofibers and these do not persist long enough to achieve therapeutic effects (78). Recently, bone-marrow cell transplantation has been envisaged as a new therapeutic avenue for DMD (80), based upon the fact that dystrophin expression was partially restored in mdx mouse muscles after intravenous

injection of bone marrow-derived stem cells (80). However, at the present time this approach yields very low incorporation of donor stem cells and would need further development before it can be considered a viable therapeutic solution for DMD.

1.5.3 Utrophin upregulation

Utrophin (formerly known as dystrophin-related protein) is closely related to dystrophin. The utrophin gene was first identified through cross-hybridisation to dystrophin cDNA (119). The complete sequence of utrophin is now known and has revealed a striking similarity with dystrophin (19). In vitro studies have demonstrated that utrophin binds to actin as dystrophin does. Utrophin also binds the DPC at the NMJ which in turn are linked to agrin in the extracellular matrix (25). Utrophin is localized at the sarcolemma in fetal muscle before being gradually replaced by dystrophin. It is speculated that utrophin may be the fetal form of dystrophin and may function in a similar way, but with dystrophin being more adapted to deal with the much greater stresses developed by skeletal and cardiac muscle after birth. In mdx mice, utrophin remains localized at the sarcolemma for a significant period after birth. Coincidentally, the signs of muscle necrosis are seen in mdx mice when utrophin becomes re-localized exclusively at the NMJ (158). The fact that utrophin can assume the same localization as dystrophin at the sarcolemma initially raised the possibility that utrophin may be capable of replacing dystrophin in myofibers (84;121;160). Subsequently, it has been demonstrated that transgenic mdx mice overexpressing truncated or full-length utrophin in dystrophin deficient muscles show amelioration or prevention (depending on the level of overexpression) of the dystrophic phenotype (46;159).

One attractive strategy that would allow exploitation of the therapeutic potential of utrophin would be to upregulate expression of the endogenous utrophin gene in DMD patients. This avenue could permit utrophin to compensate for the

missing dystrophin. Several studies aimed at characterizing utrophin gene regulatory elements are currently being performed. Thus far, it has been revealed that utrophin has a TATA-less promoter associated with CpG island (48). It contains several SP-1 sites and one N box (48). In muscle cells, the utrophin promoter responds to treatment with a nerve-derived trophic factor, heregulin, known to regulate expression of synaptic genes such as the acetylcholine receptor (75;105). The agrin gene product has also demonstrated a similar upregulation of the utrophin gene (75). These studies may have promising implications for the treatment of DMD, because they could open the door to an eventual pharmacological intervention in dystrophic muscles.

1.5.4 Gene transfer in muscle: the overview

Wolf and coworkers were the first to describe the possibility that naked DNA could be transferred into skeletal muscle by direct injection (183). Since then a great deal has been learned about ways to ameliorate the transfer of genetic material into skeletal muscles. Before being stably expressed in myofibers, the gene must cross several physical barriers. Regardless of the means of delivery (systemic or direct injection), a compulsory stop for a plasmid or viral vector is the interstitial space. However, the environment that makes up the endomysial space of muscles is often invaded with fibrosis in DMD patients (36). This could hinder the access of the transported gene into muscle fibers. A second potential physical barrier is the basal lamina, mostly consisting of a network of collagen and fibronectin. The basal lamina can restrict the passage of naked DNA because of its limited pore size (102). Beyond the basal lamina at the level of the plasma membrane itself, it has been speculated that naked DNA may be incorporated into the cell via muscle cell membrane structures called caveolae (50). Finally, once within the cell, the DNA must reach the nucleus via a mechanism that has not yet been elucidated.

Additionally, the preconditions for therapeutic success of gene transfer into DMD muscle include the following:

(i) An adequate delivery system to get the gene to the desired site of expression.

(ii) An understanding of the regulation of the gene, thus allowing persistence of expression

(iii) Ascertainment of the level of expression of the transferred gene.

(iv) Adequate outcome measures to evaluate any positive or negative changes on muscle function or systemic toxicity associated with the gene delivery.

1.5.5 Rationale for gene therapy for dystrophin-deficient muscle

While the prospect that genetic material could be readily delivered to muscle provoked a lot of enthusiasm, it had still to be determined whether dystrophin gene transfer to dystrophic muscles could prevent the development of the disease. Transgenic animal technology has allowed us to answer several questions regarding the "candidacy" of various therapeutic molecules for gene therapy of DMD (33;83;137). Experiments have reported that 50-fold overexpression of full-length dystrophin in muscles of transgenic mice prevents the development of the dystrophic phenotype (33;137). Truncated dystrophin expressed at a lower level has been shown to reach an essentially equivalent degree of therapeutic effectiveness (33;138). As mentioned earlier, development of the dystrophic phenotype was also prevented in transgenic mdx mice through overexpression of a truncated or full-length utrophin gene (46;159). Therefore, these experiments have helped to provide the foundation for the use of plasmid or viral vectors to transfer dystrophin or utrophin into muscle for treatment of dystrophin-deficiency.

1.5.6 Plasmid-based gene therapy in muscle

Plasmids containing reporter or therapeutic dystrophin genes have only offered limited expression in muscle (2). Indeed, when plasmid expressing dystrophin was injected into mdx mice, only about 1% of myofibers expressed the dystrophin gene (2). Based on transgenic mouse experiments, this level of expression was far from the 20% minimum of dystrophin-expressing myofibers required to achieve therapeutic correction in muscle (33).

1.5.7 Retrovirus-based gene therapy in muscle

Retroviruses have been used to deliver reporter or therapeutic genes to muscle. However, it appears that their intrinsic inability to infect post-mitotic cells such as myofibers will limit the wide utilization for dystrophin gene therapy. It is nonetheless worth mentioning that mdx mice injected *in vivo* with recombinant retrovirus containing a dystrophin gene could show some level of transgene expression in myofibers (165). This level expression was achieved when the delivery of recombinant retrovirus was performed in animals of 2 to 6 weeks of age (corresponding to the peak of the regenerative phase) (165). The broader use of retroviruses for dystrophic muscle has also been limited by the inherent low titer generation (50) (102).

1.5.8 Adeno-associated virus-based gene therapy in muscle

Adeno-associated virus (AAV) is a member of the parvovirus family (18). This is a non pathogenic virus that can exist in lytic or latent phase depending on the presence or absence of a coinfecting helper adenovirus (18). Wild-type AAV can integrate into the host genome and remains in latent phase at a specific site of the chromosome (18;172). Additionally, AAV has a low cellular cytotoxicity (172). Postmitotic muscle cells provide a highly permissive environment for AAV-mediated gene transfer and long-term expression (30). These features make AAV an attractive vehicle for gene therapy applications in muscle. Recently, an AAV-based vector carrying the human delta-sarcoglycan gene was successfully

employed to transduce dystrophic muscles in a hamster model of limb girdle muscular dystrophy (115). However, a major limitation of AAV is its inability to carry large genes, since its overall insertional capacity is limited to 5 kb (as opposed to the 14 kb full length dystrophin cDNA).

1.6 Adenovirus: general overview

The adenoviruses belong to the family of adenoviridae, which exist in 47 serotypes and 6 groups (A-F) based on their ability to induce tumor in hamsters (88). Infection with adenovirus in humans usually causes illnesses such as mild rhinitis, conjunctivitis, or some gastro-intestinal infections. However, some serious afflictions can be observed in immunocompromised hosts and are mainly associated with serotypes 4, 7, 11, 21 and 37 (88). The serotypes 2 and 5 (subgroup C) are currently used for gene therapy. They cause mild upper respiratory tract infection and are not associated with human malignancies.

1.6.1 Adenovirus structure

Adenovirus is icosahedrical-shaped and is made up of three structures: the nucleoprotein core, the scaffold and the outer coat of the capsid (88). This latter is composed of 720 hexons, 60 penton sand 60 trimeric fiber proteins. The scaffold protein consists of diverse polypeptides: IX, IIIa and VI whose role is to maintain the integrity of the capsid (88). The polypeptide VI relays the structural proteins to the nucleoprotein core, while the polypeptide IIIa links hexons to the adjacent face of the capsid and finally, the polypeptide IX stabilizes the packaging of the adjacent hexon subunit (88). The fiber meanwhile, protrudes from the penton bases of the capsid. This fiber mediates the attachment of adenovirus to the host cell surface, through CAR (coxsackie adenovirus receptor) (17;88). The penton base mediates the internalization of the virus by endocytosis through interaction with integrin receptors (180). The nucleoprotein complex consists of three polypeptides: u, V and VII. The terminal protein tightly bound to viral DNA is present at the 5′ extremity of the genome (88). This protein

mediates the circularization of the genome. The change of genome morphology is required for DNA synthesis (88).

1.6.2 Adenovirus genome

The adenovirus genome is composed of linear double stranded DNA of 36 kb divided into 100 map units (mu) (88). The DNA contains at each of its extremities the short inverted terminal repeats (ITRs). These ITRs mediate the circularization of the genome for viral DNA replication. The viral DNA replication is the "frontier" that separates the organization of the gene products. On one side, early gene products (E1 through E4) are expressed before the viral DNA replication. On the other side, late gene products (L1 through L5) are expressed after the initiation of viral DNA replication (88). Concerning the early genes, they are as follow: E1a, E1b, E2a, E2b, E3 and E4. E1a and E1b encode for proteins that regulate viral gene transcription. E1b has been shown to inhibit p53 function (169). Without E1, the adenovirus is unable to replicate since the expression of E1a is essential for the expression of the early genes. E2a encodes for 72 kD DNA binding protein, while the E2b encodes for the viral DNA polymerase (88). The E3 region encodes for several major proteins, including, the 19 kD that has high affinity for the molecules constituting the major histocompatibility complex class I (MHC I), which can interfere with their ability to present antigen by trapping the MHC molecules in the endoplasmic reticulum. Another product of E3 is the 14.7 kD protein which inhibits tumor necrosis factor-induced cytolysis (53;69;112;140;182). The E4 region encodes diverse gene products and is also required for the cytoplasmic accumulation of spliced mRNAs from the late primary transcripts that encode most of the virus structural proteins (151). The late region genes encode structural proteins and proteins that mediate viral assembly, packaging and finally the capsid stabilization (88).

1.7 Adenovirus-mediated gene transfer for use in dystrophin-deficient muscle

The first generation replication-deficient adenovirus that has been developed for gene therapy contains deletions of the entire E1a, a part of E1b and the E3 regions.

1.7.1 Advantages

Adenovirus-mediated gene transfer has demonstrated promise to deliver therapeutic genes to dystrophin-deficient muscles. A high level of dystrophin expressing myofibers has been reported after adenovirus-mediated dystrophin gene transfer in muscles of dystrophic mice and dogs (4;6;89;90;111). The important level of dystrophin expressing myofibers contrasted with the modest level of expression attained with plasmid-derived gene delivery in muscles (2). The relative success with regard to the level of dystrophin positive myofibers after adenovirus-based gene transfer is in part explainable by the intrinsic properties of the virus. Adenovirus is able to infect post-mitotic cells (such as myofibers) as well as dividing cells (8;109). Furthermore, the adenoviral system generates high viral titers (up to 10^{12} pfu/ml (plaque forming units) (8). By opposition to retrovirus, adenovirus does not integrate into the host genome. Thus, it is unlikely that they will alter the function of host oncogenes or tumor suppressor genes. Adding to the above-mentioned theoretical safety advantages, the clinical safety of adenovirus administration has been verified over the past 20 years, with more than ten million human subjects having received oral vaccine of live virus with no reported toxicity (162).

1.7.2 Disadvantages

1.7.2.1 Limited insertion capacity

Although adenovirus vectors offer attractive features to deliver genes to muscle, they have a limited insertion capacity. This is particularly relevant for the dystrophin gene whose the full length cDNA is 14 kb long (88). This length

exceeds the 8 kb capacity of first generation adenovirus vectors obtained with the deletion of not only E1 but also the E3 region (8;109). However, the use of a truncated version of the full-length dystrophin where 55% of the rod region is absent permits one to circumvent this drawback (139;173). In addition, the design of newer "gutless" adenovirus vectors with an insertion capacity of 36 kb has allowed for insertion of the full-length dystrophin (81;144). The gutless vector contains only the inverted terminal repeats and a packaging sequence surrounding the transgene, and all the necessary viral genes are supplemented in *trans* by a helper virus and a packaging cell line.

1.7.2.2 Immune response

a) General paradigm of the immune response to adenovirus-mediated gene transfer

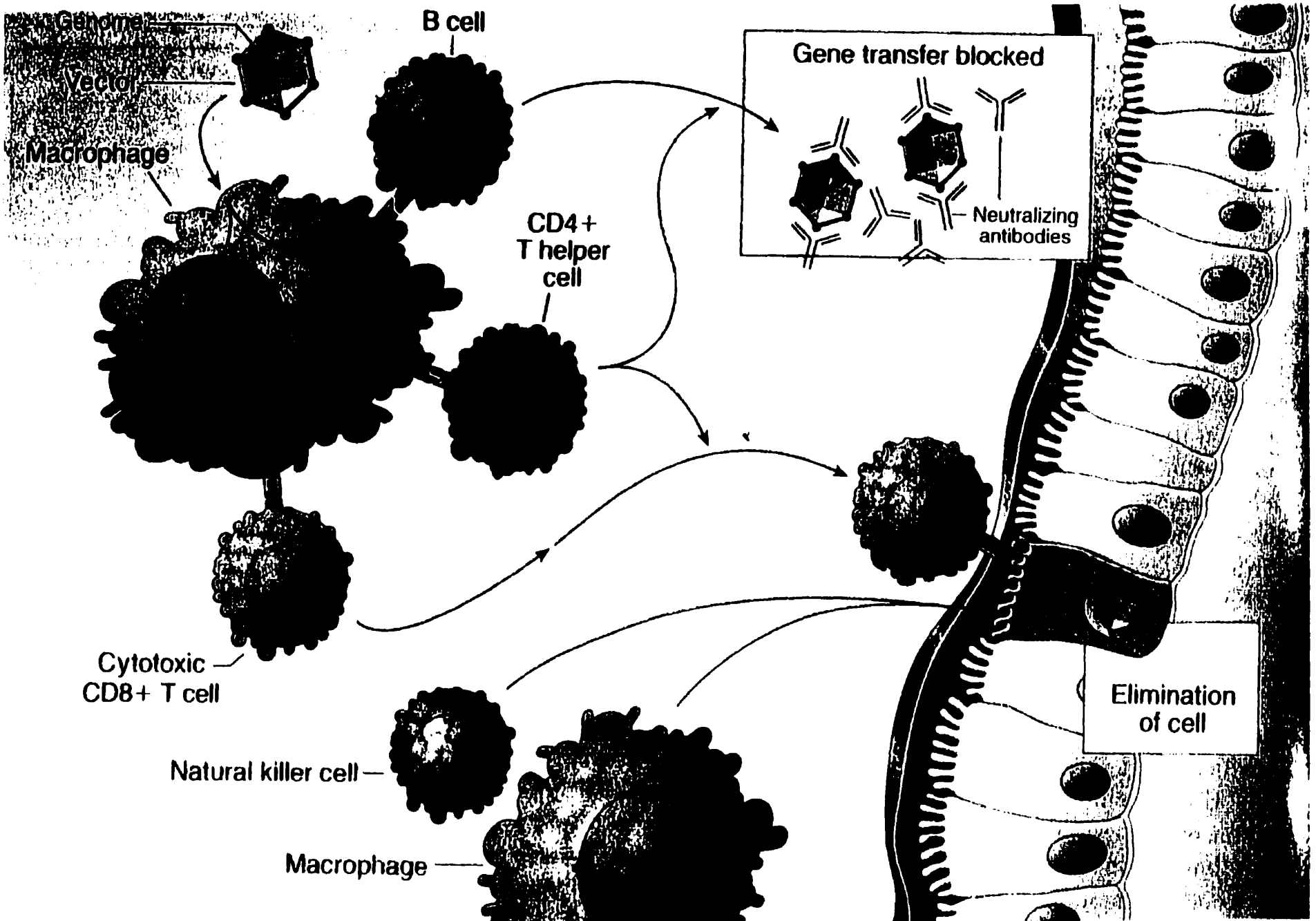
In immunocompetent adult animals, studies in different target tissues have found that transgene expression is eliminated 2 to 4 weeks after first generation AdV delivery (190;192;193). This response that peaks at 7 to 14 days has been attributed to a cytotoxic T lymphocyte (CTL) response against viral antigens presented to the surface of transduced cells by major histocompatibility complex (MHC) class I molecules (190;191). Despite the absence of the E1 region, low level "leaky" expression of adenoviral genes occurs after AdV administration (190;191). This may be related to the presence of host cell factors with E1A like activity (191;192) (58). Athymic (nude) mice fail to exhibit lymphocytic infiltrates and maintain stable transgene expression in lung and liver after AdV-mediated gene transfer (44). The use of β-2 microglobulin-deficient mice (which lack CD8+ T cells) has further supported the important role of CTLs in destroying transduced cells in the lung (190) (see figure 4).

With regard to the role of macrophages and non-antigen-specific innate immunity, they are also actors in the host immune response to AdV delivery (150;170). Macrophages are important for the processing, presentation and

opsonization of antigens that will subsequently generate specific cellular and humoral immune responses (170). Furthermore, activated macrophages have the ability to release cytokines such TNFα, IL-6, IL-12 and IFN-γ that help to prime the specific immune response (16;71). In the context of adenovirus-mediated gene transfer, they could also phagocyte AdV vectors before their entry into target cells, thereby reducing the efficacy of gene transfer. Thus, 5 days after intramuscular administration of AdV in mdx mice, injected muscles show expression of Mac-1 (a marker for macrophages) (118). Furthermore, granzyme B and IFN-δ mRNA probably produced by macrophages (or NK cells) were found to be elevated in mouse muscles 6 days after AdV-mediated gene transfer (171). Finally, macrophage depletion attenuates the rapid loss of transgene expression usually observed in immunocompetent animals and allows for a significant increase of expression of human-a1-antitrypsin (hAAt) after AdV-mediated gene transfer (110). Depletion treatment also increased the survival of animals injected with high dose of viral vector, thereby increasing the safety of adenovirus-mediated gene transfer in vivo (110).

Finally, it is worth noting that there is a difference in the assigned role of humoral and cellular immune responses. Thus, the cellular response mediates the destruction of transduced-cells, while the humoral immune response against viral particles prevents effective re-administration of the vector (118) (104;190)(see figure 4).

Figure 4. Immune responses to adenovirus vector. Dramatization of the events leading to immune-mediated inefficacy of gene therapy after adenovirus mediated gene delivery. The vector enters a macrophage, and its genome takes up residence in the nucleus. The viral proteins are presented by MHC class I molecules to CD8+ T cells. The CD4+ T cells are also activated and stimulate the action of CD8+ T cells which destroy the adenovirus transduced cells, and in the mean time activates B cells to secrete antibodies that will further neutralize the vector. These neutralizing antibodies prevent the successful readministration of the vectors [Figure taken from (181)].


Vector
Macrophage
B cell
CD4+
T helper
cell
Gene transfer blocked
Neutralizing
antibodies
Cytotoxic
CD8+ T cell
Natural killer cell
Macrophage
Elimination
of cell

b) Adenovirus-mediated gene transfer and immunogenicity in skeletal muscle

It has been now well established that neonatal muscles are more stably transduced by AdV than those of adults (3;4;6) (91). Thus, it was found that neonatal (< 5days old) mice receiving intramuscular injections of AdV showed stable reporter gene expression 2 months later, whereas gene expression was largely eliminated in adult (35-45 day old) animals by 30 days post-injection (6). Persistent dystrophin expression (up to 6 months) in a large number of myofibers was also achieved when neonatal mdx mice were injected with AdV-minidystrophin recombinant (173). These studies suggested the presence of a state of partial immunologic tolerance to AdV and the transgene when animals are injected during the neonatal period. Additionally, in adult nude or severe combined immunodeficiency (SCID) mice, long-term persistence of the transgene expression has also been attained after AdV injection into muscle (6).

The role of viral antigens in the destruction of AdV-transduced muscle fibers has been questioned (164). Indeed, several studies have reported that the immune response directed against non-viral transgene-encoded proteins is of considerable importance (128;197). It has for instance been demonstrated that antibodies produced against the LacZ transgene product is about 1000 times higher than that directed against adenoviral proteins one month after AdV-LacZ injection in muscle (135). It has also been reported that rat myofibers transfected with expression plasmids containing LacZ or luciferase transgene undergo immune-mediated destruction in a similar manner to AdV-LacZ infected cells (134). This further supports the proposition that adenoviral gene products are not necessarily the major target of the host cellular immune response . Concerning dystrophin in mdx mice or DMD patients, it represents a neoantigen that should trigger an immune response when introduced into hosts that have never been in presence of the protein. In the same line of the precedent

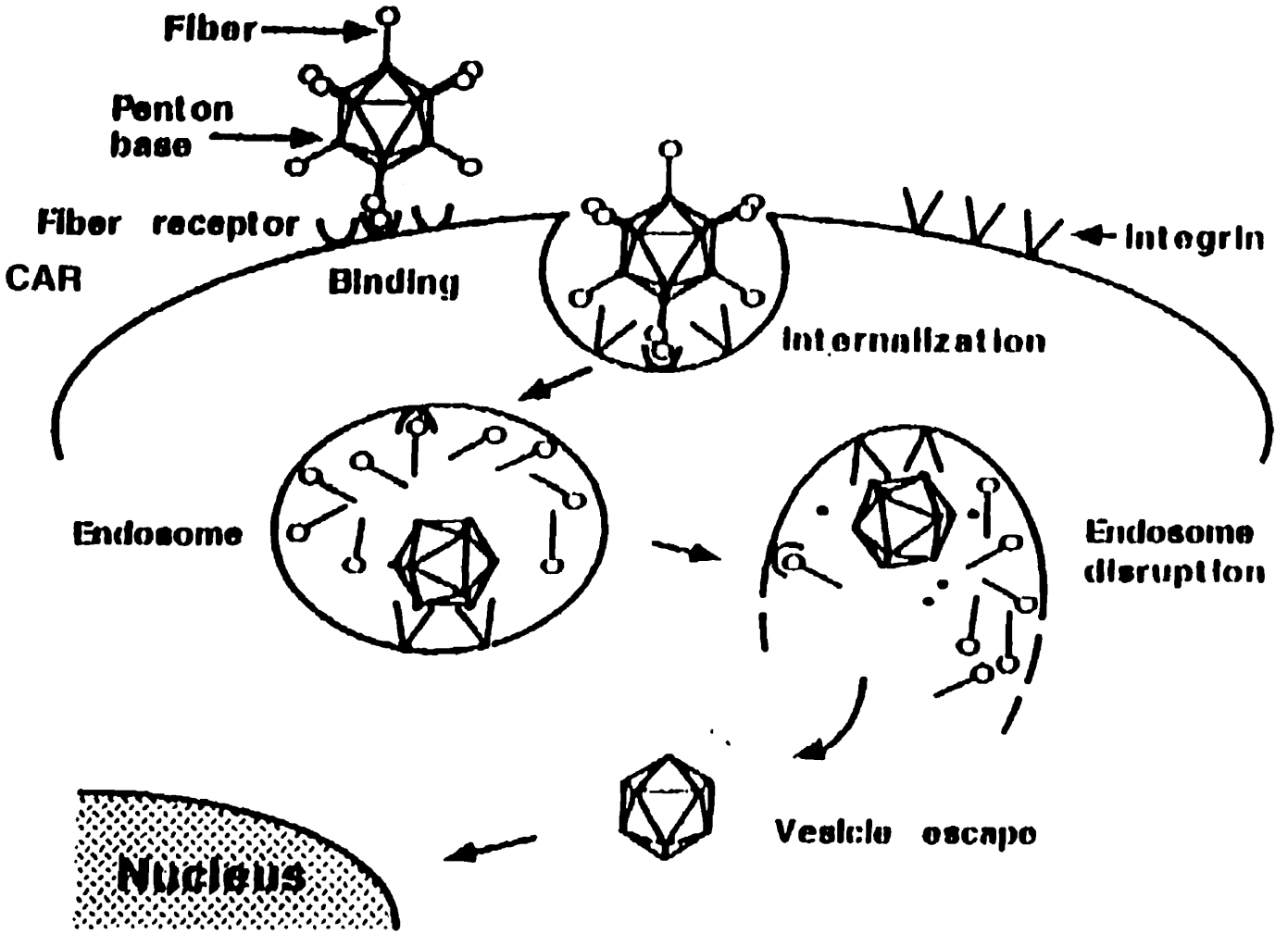
argumentation, increased levels of anti-dystrophin antibodies have been reported in mdx mice injected with AdV-dystrophin (77;118).

1.7.2.3 Maturity-dependent infectivity of adenovirus in myofibers

An additional limitation of adenoviral vectors for gene therapy in muscle is the relatively poor tropism of the virus for mature skeletal muscle (3;61;91). This limitation is at least in part related to two factors. The first one is the differential expression of the attachment and internalization receptors that mediate adenoviral uptake into the cells (61;117). The attachment is mediated through the binding of knob end of adenovirus fibers to the high-affinity Coxsackie adenovirus receptor (CAR) (17). The subsequent internalization occurs by the interaction of RGD (Arg-Gly-Asp) motifs on adenoviral coat protein and the integrin receptors (180) (see figure 5). The CAR and to a lesser extent the internalization receptor expression levels are both reduced in mature myofibers (131) (117). Thus, greater levels of adenovirus-mediated gene transfer occur in immature or regenerating muscles compared to mature muscles (29;117).

A second potential limiting factor to an efficient access of adenovirus to mature myofibers is the presence of the basal lamina (61). It has been speculated that adenoviral particles (70-100 nm of diameter) delivered in vivo, may not find their way easily to the membrane receptors due to their incapacity to cross the small pores of 40 nm within the basal lamina (102). Therefore, to the extent that the basal lamina thickness increases with the maturity of the myofibers, this may constitute another maturity-dependent factor limiting adenovirus infectivity.

Figure 5. Schematic representation of the steps leading to the expression of recombinant adenovirus in the cell. 1) The binding of the viral particle is mediated by the knob of the fibers and the linkage to CAR. Subsequently, internalization of the viral particles by endocytosis occurs, mediated by the integrin receptors. Within the endosome, proteolytic enzymes act on viral structural proteins. The vesicle is however able to escape the action of proteolytic enzymes and is ultimately released from the endosome. Thereafter the release of genetic materials from the vesicle and the targeting of viral DNA to the nucleus is able to occur (adapted from (42)).


Fiber
Penton base
Fiber receptor
CAR
Binding
Integrin
Internalization
Endosome
Endosome disruption
Vesicle escape
Nucleus

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CHAPTER 2.

Differential functional response of normal and dystrophic diaphragms after notexin-induced muscle regeneration

2.1 Prologue

As discussed in chapter 1, one of the hallmarks of dystrophin deficient muscle is its inability to regenerate effectively at the late stage of disease progression. This is ultimately reflected by the invasion of connective tissue along with an important decrease of the muscle force generating capacity. The experimental work described in the current chapter focuses on the regenerative potential of dystrophic muscle in advanced stages of disease involvement. Thus, our study emphasizes the differential regenerative responses of mdx diaphragm compared to normal diaphragm after notexin-induced muscle regeneration. We have evaluated the regenerative response of 6 months old dystrophic mouse diaphragm since this muscle exhibits a substantial degree of disease pathology that could be paralleled with the early clinical manifestations of the human dystrophy. We have hypothesized that in spite of the extent of disease progression at this age, the diaphragm can still demonstrate a regenerative response to notexin-induced muscle regeneration. Overall, the understanding and characterization of this type of response in dystrophin-deficient muscle may help to better target therapeutic intervention for DMD. This is relevant because major molecular therapies for dystrophic muscle such as myoblast transplantation or AdV-Dys delivery have been shown to require effective muscle regeneration in order to be efficient. This is then a prelude and explorative study that addresses the functional impact of notexin injection in dystrophic muscle.

2.2 Abstract

In the present study, we have induced regeneration in normal and dystrophic diaphragms of 6 months old mice using notexin, a drug with phospholipase A2 activity, known for inducing acute muscle degeneration followed by regeneration. At 20 days after the treatment, both normal and dystrophic diaphragms showed evidence of regeneration as demonstrated by histological changes in the muscle such as increased of myofiber centronucleation. The assessment of muscle contractile parameters 60 days after notexin treatment revealed that dystrophic notexinized diaphragm showed complete functional recovery. In contrast, the normal notexinized diaphragm experienced a significant loss of force-generating capacity compared to the untreated control hemidiaphragm at the equivalent recovery time point. These findings suggest that despite established signs of dystrophic pathology, experimentally-induced muscle regeneration could potentially be exploited to generate immature myofibers, the principal target of adenovirus-mediated gene transfer in muscle-directed gene therapy.

2.3 Introduction

Duchenne muscular dystrophy (DMD) is characterized by the absence of the protein dystrophin, which leads to muscle destruction and severe myofiber loss. Ultimately, the death of most patients occurs due to involvement of respiratory muscles. One of the major features of the progressive muscle deterioration in DMD is the recurrence of a degenerative (myonecrotic) process. Early in the disease progression, the degeneration is effectively compensated by muscle regeneration (2). In skeletal muscle, the regeneration observed in the post-natal period recapitulates embryonic myogenesis in many respects. The myogenic precursor cells (mpc), also called satellite cells, located between the basal lamina and plasma membrane of the myofiber are the principal actors involved in muscle repair events. Upon activation induced by injury, mpc divide and fuse to form polynucleated myotubes, which ultimately reconstitute the myofibers.

It has been suggested that in DMD after multiple cycles of degeneration and regeneration, the reparative capacity of muscle becomes exhausted due to mpc senescence (24). One of the consequences of failure of the muscle to repair itself is the invasion by connective and fat tissue. This in turn contributes importantly to loss of muscle force generating capacity (20). In the dystrophic (mdx) murine model of DMD, which also lacks dystrophin, limb muscles demonstrate relatively little fibrosis or functional alteration. However, the diaphragm of mdx mice shows histopathologic features along with functional deficits that closely resemble DMD (20).

In contrast to the situation in DMD, it has been previously reported that the regenerative potential of young mdx mice limb muscle is either greater or no different than normal muscle (9;13;14;19). However, given the phenotypic differences that exist between mdx limb and diaphragm muscles, we wished to determine whether muscle degeneration induced in the mdx diaphragm would result in effective regeneration process as previously documented for mdx limb

muscle or in a failure of regeneration with attendant loss of force-generating capacity.

Accordingly, in the present study we have evaluated the nature of the response to injury in the normal and dystrophic diaphragms of adult 6 months old mice. At this age the mdx diaphragm mouse shows clear and substantial dystrophic involvement (20). We have induced regeneration in normal and dystrophic diaphragms using notexin, a myonecrotic substance with phospholipase A2 activity. Based upon the data presented here, dystrophic diaphragms show better functional recovery compared to their normal age-matched counterparts despite a substantial level of pre-existent histopathology and functional impairment.

2.4 Materials and Methods

Animal procedures and experimental protocol: Dystrophin-deficient mdx and normal mice of the same genetic background (C57bl10) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were enrolled in the study at 6 to 7 months of age. Animals were anesthetized with intramuscular injection of ketamine (130 mg/kg) and xylazine (20 mg/kg) before the onset of the surgical procedure. The institutional animal ethics committee approved all the animal procedures described.

Induction of regeneration: A laparotomy was performed to expose the abdominal surface of the right hemidiaphragm. With the help of a dissecting microscope and forceps, the superficial fascial layer of the exposed hemidiaphragm was gently abraded. Immediately afterwards, a cotton swab immersed in notexin (10 μg/ml in 0.9% w/v NaCl saline) was applied for 30 seconds to the abraded area. The hemidiaphragm was subsequently rinsed with saline. The ability of this methodology to induce unilateral muscle injury in the rat or mouse diaphragm had previously been tested [unpublished data]. The animals were maintained on a platform tilted at an angle of about 30° toward the targeted hemidiaphragm to avoid diffusion of notexin to the untreated side. The animals continued to breathe spontaneously during the procedure. Animals recovered well from surgery with no apparent untoward side effects.

Measurement of isometric contractile properties: At 60 days after the induction of muscle regeneration, isometric contractile properties were examined. Mice were euthanized by overdose of anesthetic. The diaphragm was quickly removed and transferred to chilled Ringer's solution (composition 119 mM NaCl, 4.7mM KCl, 2.5mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 20 mM NaHCO3) perfused with 95% 02: 5%CO2 (pH 7.4). Muscle strips were dissected from each hemidiaphragm for evaluation contractility parameters. Muscles were

then mounted in random order into a jacketed tissue bath chamber continuously perfused with Ringer's solution, containing 6 μM d-tubocurarine and maintained at 25°C. A thermo-equilibration period of 15 min was observed before initiating contractile measurements. One end of each muscle was securely anchored to a platform near the base of the chamber, while the opposite tendon was tied to the lever arm of a force transducer/length servomotor system (model 300B dual mode; Cambridge Technology, Watertown, MA, USA). The latter was mounted on a mobile micrometer stage (Newport Instrument, Toronto, Canada) to allow incremental adjustment of muscle length. Electrical field stimulation was induced via platinum plate electrodes placed into the bath on both sides of the muscle. Supramaximal stimuli with a monophasic pulse duration of 2 ms were delivered using a computer-controlled electrical stimulator (model S44, Grass Instruments, Quincy, MA, USA) connected in series to a power amplifier (model 6824A; Hewlett Packard, Palo Alto, CA, USA). Muscle force was displayed on a storage oscilloscope (Tektronix, Beaverton, OR, USA), and the data were simultaneously acquired to computer (Labdat/Anadat Software; RHT-Infodata, Montreal, Canada) via an analog-to-digital converter at a sampling rate of 1000 Hz for later analysis. After adjusting each muscle to optimal length (Lo, the length at which maximal twitch force is achieved), five twitch stimulations were recorded and the mean value was used to determine the following: maximal isometric twitch force (Pt), twitch contraction time (CT), twitch half relaxation time (HRT). The tetanic force was evaluated at the following stimulation frequencies: 10, 30, 60,90 and 120 Hz. The maximal isometric tetanic force (Po) was then defined as the maximal force measured during generation of the force-frequency curve. The muscle was removed from the bath and Lo was directly measured under a dissecting microscope with calipers accurate to 0.1 mm. Total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.06 g/cm3). This allowed specific force to be calculated, which was expressed as N/cm2.

Hematoxylin and eosin, centronucleation index: After the completion of the muscle function studies, the diaphragm was embedded in mounting medium and snap-frozen in isopentane pre-cooled with liquid nitrogen. Transverse sections 6μm thick were obtained in a cryostat and then stained with hematoxylin and eosin (H & E) to allow determination of the prevalence of centrally nucleated myofibers. The proportion of myofibers with centronucleation is used as an indicator accumulated injury and subsequent regeneration (11;16). Microscopically visualized sections were photographed using a video camera and the image was stored on a Macintosh computer. The counting of total number of myofibers and the centrally nucleated myofibers was performed on each section using the public domain program NIH Image (version 1.49). The centronucleation index was defined as the ratio of the number centrally nucleated myofibers to the total number of myofibers on the same tissue section.

Embryonic myosin heavy chain (MHC): Serial sections were collected and reacted with a monoclonal antibody specific to the embryonic isoform of myosin heavy chain (MHC), diluted 1:37.5 in PBS as previously described (17). This was followed by incubation with biotinylated goat anti-mouse secondary antibody (1:150). The visualization of the myofibers expressing embryonic MHC was done using peroxidase staining with the Elite Vectastain ABC kit (Vector Laboratories, Mississauga, Ontario).

Statistical analysis

Within each group, differences between notexin-treated and control (untreated) hemidiaphragms were assessed by using a paired-t test. The significance level was set at $p<0.05$.

2.5 Results

Short-term effect of notexin-induced regeneration on normal and dystrophic diaphragms

Diaphragm morphology: At 20 days after notexin injection, the normal diaphragm shows many myofibers with central nucleation (figure 1a). In the mdx diaphragm, a high level of central nucleation is also present, along with other sings of the underlying dystrophic process such as increased inflammation and connective tissue (figure 1b). The staining for embryonic MHC in normal muscle 20 days after induction of regeneration shows no reactivity (data not shown). In mdx muscle, although occasional foci of regeneration can be noticed, the bulk of the regenerative process is also over as evidenced by a low level of embryonic MHC staining (figure 1c).

In order to assess more quantitatively the regenerative process, we have analyzed the proportion of centrally nucleated myofibers in the different groups. The centronucleation index is a quantitative indicator of regenerated myofibers (11;16). The normal diaphragm exposed to notexin shows a significant increase in the centronucleation index compared to untreated side (figure 2). Given the pre-established presence of centronucleation in dystrophic diaphragms the increase in the centronucleation index after notexin exposure is less accentuated for the mdx group but nonetheless present, although not a statistically significant level (figure 2).

Long-term effect of notexin-induced regeneration on normal and dystrophic diaphragms

Diaphragm morphology: At 60 days after the induction of regeneration, the normal muscle exposed to notexin shows persistent centrally nucleated myofibers (figure 3b). In mdx animals the morphology of the diaphragm 60 days

after notexin appears similar to the control (non-notexined) mdx hemidiaphragm. (figures 3c & 3d).

Diaphragm contractile kinetics and isometric force: Kinetics of the normal diaphragm at 60 days after regeneration demonstrated a trend toward reduction of the contraction and half relaxation times for the notexin-treated side (Table 1). A similar tendency was seen in the mdx group at 60 days after notexin exposure (table 1). Additionally, analysis of the force generating capacity for normal diaphragm demonstrated a significant depression of maximal tetanic force in notexin-treated hemidiaphragms compared to the untreated side (Table 2). On the other hand, the mdx group demonstrated no significant change in maximal isometric force in notexin-treated hemidiaphragms (Table 2). Examination of the force-frequency relationship confirms the net depression of force in notexinized normal diaphragm compared to the untreated side (figure 4a). However, there was no significant alteration by notexin of specific force across the force-frequency relationship in the mdx group as illustrated in figure 4b.

Figure 1. Efficiency of regeneration at short-term. **(A)** H&E stained cross-section of diaphragm of C57BL10 mouse, at 20 days after induction of regeneration with notexin. Note that most of the fibers exhibit centronucleation, indicating that they had previousiy undergone a cycle of degeneration and regeneration. **(B)** Cross-section of dystrophin deficient mdx mouse diaphragm 20 days after the induction of regeneration with notexin. Most the myofibers exhibit centro-nucleation, a feature which characterizes dystrophic muscle. **(C)** Serial section of dystrophic diaphragm immunoreacted with embryonic MHC antibody 20 days after induction of regeneration with notexin. Most of the myofibers are negative for embryonic MHC. However, some foci of regenerating myofibers are observed (star). The normal mouse hemidiaphragm immunoreacted with the same antibody is completely negative for the embryonic MHC staining (data not shown).

Figure 2. Quantitative evaluation of the level of notexin-induced muscle regeneration in the diaphragm, using the index of centronucleation in normal and dystrophic muscle. There was a significant increase in the centronucleation index in notexinized non-dystrophic hemidiaphragms compared to the untreated side, whereas the augmentation of the centronucleation index in dystrophic hemidiaphragms is less accentuated and non significant. The values represent means ± SE, * $P<0.05$ versus the untreated (non-notexinized) hemidiaphragm. (n=5 and n=6 for bl10 and mdx, respectively)

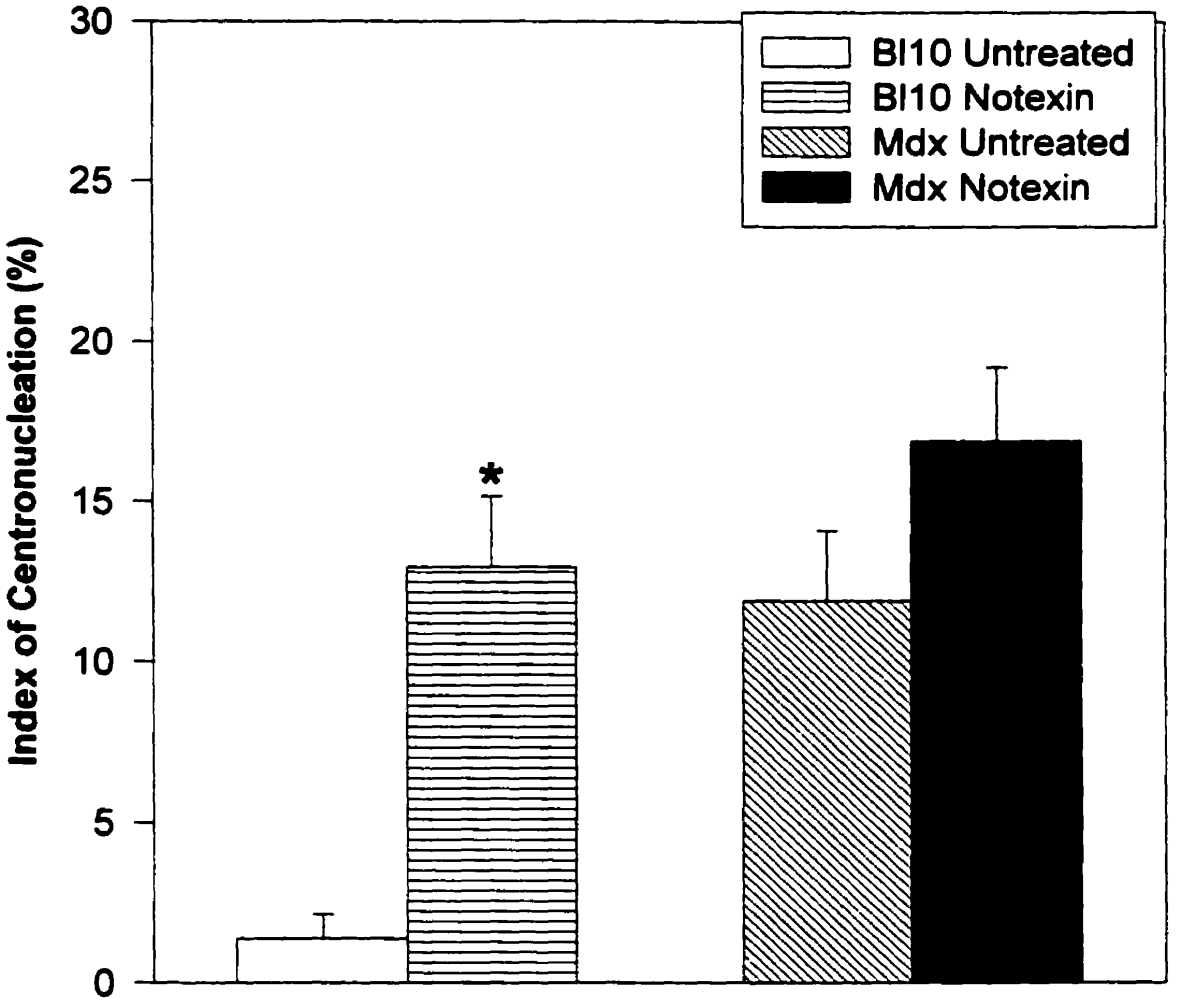

30
25
20
15
10
5
0
Index of Centronucleation (%)
*
Bl10 Untreated
Bl10 Notexin
Mdx Untreated
Mdx Notexin

Figure 3. H & E staining of the diaphragm 60 days after notexin-induced muscle regeneration. **(A)** The untreated normal hemidiaphragm shows myofibers with no apparent histological abnormality. **(B)** The notexinized normal hemidiaphragm presents myofibers with centronucleation. **(C)** The untreated dystrophic hemidiaphragm harbors standard dystrophic alterations with centronucleation and connective tissue invasion. **(D)** The notexinized dystrophic hemidiaphragm presents the similar alterations observed in (c).

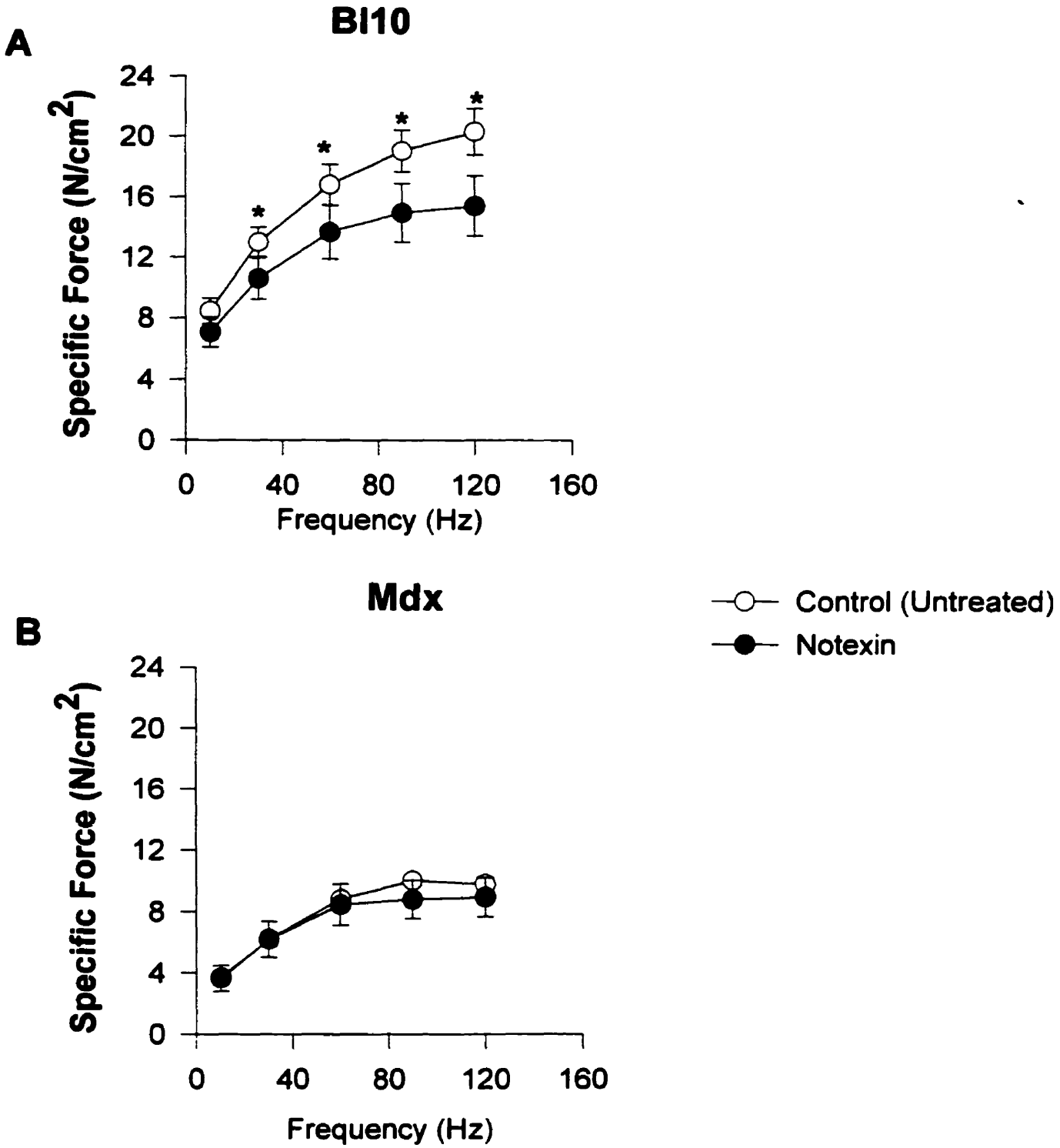
Table 1 Long-term (60 days) effects of notexin-induced muscle regeneration on normal and dystrophic diaphragm contractile kinetics. The values represent means ± SE.

Groups *(mice)*	n	Contraction Time *(ms)*	Half Relaxation Time *(ms)*
C57BL10	5		
Untreated		43.6±4.2	52.0±7.0
Notexin-treated		37.2±3.5	38.8±3.2
MDX	5		
Untreated		48.0±7.4	53.0±4.5
Notexin-treated		42.5±4.5	42.5±3.5

Table 2. Long-term (60 days) effects of notexin-induced muscle regeneration on normal and dystrophic diaphragm maximal isometric force generation. The values represent means ± SE. * P<0.05 versus the untreated hemidiaphragm.

Groups *(mice)*	n	Maximal Twitch *(N/cm2)*	Maximal Tetanus *(N/cm2)*
C57BL10	5		
Untreated		5.7±0.4	20.4±1.5
Notexin-treated		4.3±0.6	15.4±1.9*
MDX	5		
Untreated		2.1±0.6	10.1±0.3
Notexin-treated		2.4±0.7	9.4±1.4

Figure 4. The diaphragm force-frequency relationship in normal **(A)** and dystrophic **(B)** mice at 60 days after notexin-induced-muscle regeneration. There was a significant decrease in specific force in notexinized normal hemidiaphragms compared to the untreated side. There was no change of specific force in notexinized dystrophic hemidiaphragms. The values represent means ± SE, (n=5 for both normal and dystrophic mice) * $P<0.05$.


A
BI10
Specific Force (N/cm2)
24
20
16
12
8
4
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40
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Frequency (Hz)
*
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*
B
Mdx
Specific Force (N/cm2)
24
20
16
12
8
4
0
0
40
80
120
160
Frequency (Hz)
Control (Untreated)
Notexin

2.6 Discussion

The major finding of the present study is that despite advanced pre-existent dystrophic pathology and functional impairment, the mdx mouse diaphragm demonstrated complete functional recovery of force-generating capacity by approximately 2 months after experimentally-induced injury. In contrast, the normal non-dystrophic diaphragm showed a persistent force deficit compared to baseline values when subjected to the same type of injury.

This is the first study to examine functional recovery in the mdx diaphragm after experimentally-induced injury and subsequent regeneration. Previous studies have examined functional responses to injury in limb musculature of younger mdx animals (13;14), which have less in the way of pre-existent histopatholgical and functional abnormalities. In this regard, Mechalchuk and Bressler (13) reported that at 13 wks after injury and regeneration induced by denervation/devascularization, mdx extensor digitorum longus (EDL) muscles showed a return of absolute as well as weight-normalized force to baseline pre-injury levels, whereas these parameters were persistently depressed in the non-dystrophic EDL at the same time point. Moens et al. (14) reported that 2 months after isotransplantation of the soleus to induce a cycle of complete degeneration/regeneration, there was a persistent but equivalent force deficit (amounting to approximately 40%) in both groups.

In keeping with the above functional data, previous histological analyses have also suggested that the regenerative response to injury is either equivalent or superior in mdx as compared to non-dystrophic hindlimb muscles (1;9;10;19;25). For instance, when the myonecrotic agent bupivicaine was repeatedly injected into the soleus muscles of 7 wk-old non-dystrophic and mdx mice, there was no significant difference in the level of muscle collagen accumulation between either group (10). Similarly, after crush injury imposed on the tibialis anterior muscles

of 5-7 wk-old mdx and normal non-dystrophic mice, there was also no measurable difference in the histological appearance of regenerated lesions nor in the level of activation of muscle precursor cells (9).

In the present study, increased centronucleation over baseline (pre-notexin) values allowed confirmation that necrosis and subsequent regeneration occurred. In contrast, the trend toward increased centronucleation in the mdx diaphragm after notexin did not attain statistical significance. This finding raises the possibility that the dystrophic diaphragm could be more resistant in some way to the necrotic effects of notexin. However, many studies have demonstrated the effectiveness of notexin in producing necrosis in mdx limb musculature (8;12). In addition, our findings are consistent with data obtained using physical models of degeneration/regeneration such as denervation/devascularization, in which the centronucleation index was not further increased above baseline levels in mdx muscles that had already undergone spontaneous episodes of necrosis and regeneration prior to the imposition of experimental injury (19;25). This is likely due at least in part to the fact that degeneration/regeneration induced by experimental injury also occurs in myofibers with pre-existent centronucleation. In addition, it has been suggested that certain pools of muscle precursor cells recruited to the site of injury tend to result in peripheral nucleation of regenerated myofibers, whereas others are more likely to produce central nuclei (19). Therefore, there could conceivably be differential recruitment of muscle precursor cell subpopulations after induced regeneration in the two strains of mice.

The fact that there was incomplete functional recovery of the normal non-dystrophic diaphragm at 60 days post-notexin is consistent with previous studies in limb muscles subjected to bupivicaine (5;18) or muscle transplantation (4) models of degeneration/regeneration. The superior functional recovery of the mdx diaphragm in this study may suggest two major possibilities: 1) an

inherently greater regenerative capacity of the mdx diaphragm compared to the non-dystrophic diaphragm; and 2) a protective effect of experimentally-induced regeneration against the damaging effects of dystrophin deficiency. With regard to the former, Anderson et al. (3) found no evidence for a superior proliferative capacity of mdx diaphragm myoblasts compared to myoblasts from normal non-dystrophic diaphragms, although there was some suggestion of reduced proliferative potential in mixed (myoblast and fibroblast) diaphragm cell cultures as compared to limb.

On the other hand, a possible increase in the resistance of regenerated mdx diaphragm myofibers to subsequent spontaneous episodes of necrosis caused by the underlying disease process is supported by several lines of evidence. Firstly, after an initial period of widespread muscle necrosis followed by regeneration in early life, the level of necrosis tapers off to a persistent but low level in most mdx muscles (7). Second, the dystrophin-related protein known as utrophin, which is capable of acting as a functional surrogate for dystrophin in mdx muscles (21-23), is transiently upregulated in regenerated fibers. Lastly, it has recently been demonstrated that normal rat limb muscle subjected to bupivicaine-induced degeneration/regeneration shows a markedly reduced susceptibility to force deficits and histological injury induced by high-stress lengthening (eccentric) contractions (6). It is hypothesized that the sarcomeres of regenerated fibers possess greater homogeneity in their mechanical strength properties, which would make individual sarcomeres in series less likely to undergo mechanical failure during lengthening contractions (15).

In summary, the present investigation has demonstrated that in the dystrophin-deficient diaphragm, the superimposition of experimental injury in this muscle with advanced dystrophic pathology and functional impairment does not lead to any worsening of contractile function. Because experimentally-induced regeneration of this type has been shown to improve the efficiency of different

strategies for dystrophin gene transfer (e.g., using viral vectors or cell therapy), these findings may have implications for eventual gene-based therapies in DMD patients.

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CHAPTER 3.

Functional consequences of adenovirus-mediated minidystrophin gene transfer in experimentally regenerated muscles of old dystrophic (mdx) mice

3.1 Prologue

In the preceding chapter, we examined the regenerative and functional response of dystrophic muscle with advanced disease to experimental induction of regeneration with notexin. In the current chapter, we have taken advantage of the inherent regenerative potential of dystrophic to deliver an adenovirus vector carrying a dystrophin minigene (AdV-Dys). Here we have accomplished induction of regeneration in limb muscles of old (14-17 months) mdx mice. The limb muscles of old mdx mice show a substantial degree of dystrophic pathology (similar to the diaphragm at 6 months of age). We demonstrate that the use of notexin in old dystrophic limb muscles generates numerous immature myofibers that overexpress the Coxsackie adenovirus receptor (CAR), the high affinity attachment receptor required for efficient adenovirus infection. Accordingly, we have delivered AdV-Dys into these regenerated muscles and found a significantly increased level of AdV-mediated dystrophin gene transfer comparatively to non-regenerated old mdx limb muscles. Therefore, the manipulation of the host muscle carried out in the present investigation has enabled us to assess:

1) The upregulation of CAR on regenerated myofibers in old dystrophic limb muscles with advanced pathology;
2) The risk:benefit ratio associated with the induction of regeneration in old mdx muscles, either alone or combined with AdV-Dys delivery;
3) The therapeutic effects associated with sustained expression of dystrophin in muscles of old mdx with substantial pre-existent dystrophic signs.

3.2 Abstract

Adenovirus vector (AdV)-mediated dystrophin minigene transfer has produced functional benefit when performed in young adult and neonatal mdx dystrophic mice. However, AdV-mediated gene delivery to muscles of old mdx animals is considerably less efficient, related at least in part to low levels of the Coxsackie and adenovirus attachment receptor, CAR. In young mdx mice, administration of the myonecrotic agent notexin provokes muscle regeneration through a recapitulation of myogenesis, which leads to an increase in immature myofibers that overexpress CAR. Accordingly, in this study we have examined whether notexin-induced muscle regeneration can be similarly used to upregulate CAR expression and thereby increase the efficiency of AdV-mediated dystrophin gene transfer in old mdx mice (14-17 months) with advanced dystrophic pathology. At five days after notexin injection, numerous fibers coexpressed embryonic myosin heavy chain and CAR. The delivery of AdV-Dys five days after notexin injection led to significantly augmented dystrophin expression compared to muscles that did not receive notexin prior to AdV-Dys administration. However, although the use of notexin alone did not result in any short or long-term deleterious effects on muscle function, the combined utilization of notexin and AdV-Dys in old mdx muscles produced a transient decrease in muscle cross-sectional area. Importantly, by 50 days post-AdV a substantial level of dystrophin expression persisted, and old mdx muscles serially treated with notexin and AdV-Dys showed recovery of muscle cross-sectional area as well as improved force-generating capacity compared to non-treated muscles. Therefore, it appears that the long-term risk:benefit ratio associated with the use of experimentally-induced regeneration followed by AdV-mediated dystrophin gene transfer is favorable in old mdx mice despite the presence of advanced dystrophic pathology. These findings may have implications for AdV-mediated dystrophin gene transfer in human DMD patients.

3.3 Introduction

Adenoviral vectors (AdV) are extensively used to deliver transgenes to numerous tissues including skeletal muscle (2;3) Their utilization in skeletal muscles of mdx mice, the murine homologue of Duchenne muscular dystrophy (DMD), has permitted a high level of reporter and therapeutic dystrophin transgene expression to be achieved (13;17;22;25;27;31;39). The relative success obtained for gene delivery in mdx mouse skeletal muscles is attributable in part to the intrinsic characteristics of the vector such as an ability to transfect post-mitotic cells and be produced in high titer (21). Characteristics of the promoter driving expression of the transgene have also been shown to play an important role in the efficiency of gene expression (18). In addition, the efficiency of adenoviral transduction in myofibers is highly dependent upon the state of maturation of the muscle (1;11;34). In this regard, successful AdV-mediated gene transfer is most readily achievable in immature myofibers, which are prevalent in neonatal and young adult mdx mice (17) but rare in older mdx animals (10).

The relatively low efficiency of AdV-mediated gene transfer in mature muscle fibers appears to be related, at least in part, to low levels of the high-affinity Coxsackie and adenovirus attachment receptor (CAR) (5;29). Thus, whereas young mdx muscles harbor a substantial subpopulation of myofibers with immature phenotypic characteristics including relative upregulation of CAR expression, in older mdx muscles CAR is downregulated in conjunction with the overall reduction in muscle fiber regeneration (10;29). In young mdx mice, CAR expression and the efficiency of AdV-mediated gene transfer can be further enhanced by pre-treating muscles with notexin, which provokes muscle fiber necrosis through its phospholipase A2 activity, followed by a vigorous regenerative response with attendant upregulation of CAR (7).

It is unknown whether a similar strategy could be successfully employed in dystrophic muscles with more advanced pathology. Because patients with DMD

are generally diagnosed at a point in their disease which more closely resembles the advanced pathology seen in old mdx mice, the latter probably represents a more realistic representation of the anticipated clinical scenario as compared to the neonatal or young adult mdx mouse models. However, it has been suggested that muscles of old mdx mice (4;23;30;41) as well as DMD patients (6;36) have a significantly reduced ability to regenerate in response to induced injury. Therefore, there is a concern that the use of notexin or other similar agents to induce regeneration in this setting could result not only in a lack of effective CAR upregulation, but also in deleterious effects on muscle repair with consequent aggravation of the already substantial muscle weakness.

Accordingly, the specific objectives of the present investigation were 3-fold: 1) to determine whether notexin administration to old mdx muscles with advanced pathology leads to effective CAR upregulation and an increased efficiency of AdV-mediated dystrophin gene transfer; 2) to ascertain whether notexin administration in this setting leads to adverse effects on muscle function; and 3) to evaluate the therapeutic efficacy of combinatorial treatment with notexin and AdV-Dys as a means of improving muscle strength in old mdx mouse muscles.

3.4 Materials and Methods

Preparation of recombinant adenovirus vector

Adenovirus recombinants containing 6.3-Kb human dystrophin minigene (AdV-Dys) were constructed by using E1/E3-deleted replication-defective serotype 5 human adenovirus as previously described, where the transgene cDNA was driven by cytomegalovirus promoter/enhancer elements inserted into the E1 region (26). The absence of contamination by E1-containing replication-competent AdV was confirmed by using a sensitive PCR screening assay as previously described (26). AdV titers were determined by spectrophotometry at 260 and are expressed as particles per milliliter.

Animal procedures

Dystrophin-deficient mdx mice were purchased from The Jackson laboratory (Bar Harbor, Maine) and enrolled into the study at 14-17 months of age. Prior to notexin or AdV injection, the mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg) into the forelimb. Hindlimb muscles were then surgically exposed to permit notexin and AdV-Dys injections under direct visualization with the aid of dissecting microscope. Targeted muscles consisted of either the tibialis anterior (TA) or soleus; these muscles were selected to allow comparisons with previous studies from our laboratory (2;7;39).

In order to prevent immune-mediated destruction of AdV-infected myofibers, all animals were injected with FK506 (3mg/kg/day subcutaneously), a potent inhibitor of activated T-lymphocyte proliferation (25). This immunosuppressive regimen was selected based upon our previous demonstration of sustained high-level of dystrophin expression 2 months after AdV-Dys delivery to mdx mice (25). Immunosuppression was begun on the day before AdV-Dys injection and continued until the animals were sacrificed at the end of the designated experimental period. The institutional animals ethics committee approved all procedures.

Muscle regeneration protocols

a) Effects of regeneration on CAR expression and myofiber transduction with AdV-Dys: Notexin (Sigma, Oakville, Canada) was administered by a single injection (10 µl of 10 µg/ml solution in 0.9%w/v saline) into the right TA muscle, while the contralateral side received the equivalent volume of saline. Notexin administration to normal muscle at this dose has been shown to elicit myofiber necrosis without damaging the basal lamina, nerves or blood vessels, which then allows for effective muscle regeneration (16;24). At 5, 20 and 55 days later, TA muscles from both legs were removed for immunohistochemical detection of CAR and the embryonic isoform of myosin heavy chain, a marker of muscle regeneration (see below). To evaluate the effects of notexin-induced regeneration on the efficiency of myofiber transduction by AdV, TA muscles were treated as described above (right side: notexinized; left side: saline) and 5 days later 15 µl of AdV-Dys (OD titer 1 X 10^{11} particles per ml) was injected into both muscles. This time point was based upon preliminary data indicating maximal CAR expression. At 15 and 50 days after AdV-Dys injection, animals were euthanized and immunostained to evaluate dystrophin expression.

b) Effects of notexin-induced regeneration on muscle function: In order to evaluate the effects of notexin alone on muscle function in old mdx mice, 5 µl of notexin (10 µg/ml in 0.9%w/v saline) was injected into the left soleus, while the right soleus received the equivalent volume of saline. At 20 days and 55 days after notexin administration (corresponding to the same time lapse post-notexin described above), animals were euthanized and soleus muscles were removed for *in vitro* contractility measurements and immunohistochemical analysis.

c) *Effects of notexin pre-treatment and subsequent AdV-Dys delivery on muscle function:* In order to evaluate the combined effects of notexin-induced muscle regeneration and AdV-Dys delivery, the left soleus was injected

with notexin as described above in part b, and 5 days later 10 µl of AdV-Dys (OD titer 1 X 10^{11} particles per ml) was delivery into the same muscle. The contralateral muscle was sham-treated with the equivalent volume of saline. At 15 and 50 days after AdV-Dys injection, animals were euthanized and soleus muscles were removed for *in vitro* contractility measurements and immunohistochemistry.

Measurement of isometric contractile properties

Mice were first anesthetized to achieve a loss of deep pain reflexes. Soleus muscles were then carefully removed in random order and tested to assess *in vitro* isometric contractile properties as follows. Freshly dissected soleus muscles were briefly transferred to chilled Ringer's solution (composition 119 mM NaCl, 4.7mM KCl, 2.5mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 20 mM NaHCO3) perfused with 95% 0_2: 5%CO_2 (pH 7.4). The muscles were then mounted vertically in a jacketed tissue bath chamber perfused continuously with Ringer's solution containing 6 µM d-tubocurarine and maintained at 25°C. A thermoequilibration period of 15 min was observed before initiating contractility measurements. One end of each muscle was securely anchored to a platform near the base of the chamber, while the opposite tendon was tied to the lever arm of force transducer/length servomotor system (model 300B dual mode; Cambridge Technology, Watertown, MA, USA). The later was mounted on a mobile micrometer stage (Newport Instrument, Toronto, Canada) to allow incremental adjustment of muscle length. Electrical field stimulation was induced via platinum plate electrodes placed into the bath on both sides of the muscle. Supramaximal stimuli with monophasic pulse duration of 2 ms were delivered using a computer-controlled electrical stimulator (model S44, Grass Instruments, Quincy, MA, USA) connected in series to a power amplifier (model 6824A; Hewlett Packard, Palo Alto, CA, USA). Muscle force was displayed on a storage oscilloscope (Tektronix, Beaverton, OR, USA), and the data were simultaneously

acquired to computer (Labdat/Anadat Software; RHT-Infodata, Montreal, Canada) via an analog-to-digital converter at a sampling rate of 1000 Hz for later analysis.

After adjusting each muscle to optimal length (Lo, the length at which maximal twitch force is achieved), five twitch stimulations were recorded and the mean value was used to determine the following: maximal isometric twitch force (Pt), contraction time (CT), twitch half relaxation time (HRT). Tetanic force was evaluated at the following stimulation frequencies: 10, 30, 60, 90, and 120 Hz. The maximal isometric tetanic force (Po) was then defined as the maximal force measured during generation of the force-frequency relationship. The muscle was then removed from the bath and Lo was directly measured under a dissecting microscope with calipers accurate to 0.1 mm. Total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.06 g/cm3). This allowed specific force (normalized to cross-sectional area) to be calculated, which was expressed as N/cm2.

Response to high-stress lengthening contractions

Dystrophin is postulated to play an important role in helping to protect myofibers from potentially damaging mechanical stress associated with muscle contraction (32;37). Therefore, after allowing a 10 min recovery period following the above measurement of isometric contractile properties, soleus muscles were further studied to determine their ability to withstand high-stress lengthening (eccentric) contractions. The protocol has been previously described in detail (32). Briefly, the soleus was supramaximally stimulated at the frequency achieving Po (maximal tetanic force) for 700 ms; the muscle was held at Lo during the initial 500 ms, whereas a step-change increase in length of 15% Lo was imposed by the computer-controlled servomotor lever arm during the final 200 ms of stimulation. A total of five such stimulations was performed, each being separated by a 2 min recovery period at Lo. The decline in maximal isometric

force (obtained from the first 500 ms of muscle stimulation at Lo) during successive contractions was then recorded and used as an indicator of contraction-induced myofiber injury as previously described (9;32). This index of contraction-induced myofiber injury was then normalized by the maximal isometric tetanic force attained to adjust for the level of stress applied to the muscle.

Immunohistochemistry

The soleus and TA muscles were embedded in mounting medium and snap-frozen in isopentane pre-cooled with liquid nitrogen.

a) Coxsackie/adenovirus Attachment Receptor (CAR): Transverse cryostat sections (5 μm thick) were fixed in 1% acetone. Anti-CAR antibody (29) was used at a dilution of 1:40 diluted in a blocking solution (3% BSA, 0.05% Tween-20 in PBS). Immunoreactivity was revealed by adding a secondary antibody with Cy3-conjugated affinity-purified anti-rabbit IgG (1:100 Jackson Immunoresearch Laboratories, West Grove, PA).

b) Embryonic Myosin Heavy Chain (MHC): Serial sections (aligned with the precedent CAR staining) were collected and reacted with monoclonal antibody specific for the embryonic isoform of myosin heavy chain (MHC) diluted 1:37.5 in PBS as previously described (33). This was followed by incubation with biotinylated goat anti-mouse secondary antibody (1:150). The visualization of myofiber immunoreactivity was done via peroxidase staining using the Elite Vectastain ABC kit (Vector Laboratories, Mississauga, Ontario).

c) Dystrophin: Transverse sections (5 μm thick) were obtained from the midportion of the muscle and then fixed on slides in 1% acetone. Immunohistochemical procedures were carried out as previously described to detect dystrophin expression using a polyclonal anti-dystrophin (C-terminus)

primary antibody and biotinylated secondary antibody followed by visualization with Cy3-conjugated streptavidin.

Statistical analysis

To determine whether a significant difference between the notexin-treated muscles and the contralateral control was reached, a paired-test was performed at each time point. To assess differences within the same treatment group at the two different time points studied, a t-test for unpaired samples was performed. The significance level was set at $p < 0.05$.

3.5 Results

Efficiency of regeneration and CAR expression in old mdx muscles after notexin injection

Because it has been previously shown that old mdx mice experience a loss of myogenic capacity that is similar to what is seen in DMD (4;23;30;41)., we were first interested in assessing whether effective regeneration can still be produced by notexin in muscles of elderly mdx mice with advanced disease. Tibialis anterior (TA) muscle sections immunostained for embryonic myosin heavy chain (MHC) are shown in figures 1a & 1c. A scanty presence of regenerating myofibers can be observed in the non-notexinized TA muscle (figure 1a), whereas numerous regenerating fibers with intense cytoplasmic embryonic MHC staining are noted in the contralateral TA muscle 5 days after notexin injection (figure 1c). Five days after induction of regeneration, notexin-treated muscles also demonstrate intense CAR immunoreactivity (figure 1d), both at the level of the sarcolemma and within the cytoplasm. Note that there is a substantial correlation between fibers expressing embryonic MHC and CAR (figures 1b & d). The intensity of CAR expression in myofibers at 20 days after notexin-induced regeneration was importantly reduced but could still be seen, whereas by 55 days post-notexin CAR expression has returned to baseline levels similar to that seen in figure 1b.

Efficiency of AdV-mediated dystrophin gene transfer in old mdx muscles after regeneration induced by notexin

Since high CAR expression was found in regenerating immature myofibers 5 days after notexin injection, this time point was selected for delivery of recombinant adenovirus containing the dystrophin minigene (AdV-Dys). As shown in figures 2 & 3, there were significantly more dystrophin-expressing myofibers in TA muscles pre-treated with notexin comparatively to saline pre-treated control muscles at both 15 and 50 days after AdV-Dys administration. Furthermore, under the immunosuppression regimen used there was no significant loss of

dystrophin positive myofibers in either group between the two time points examined (P=0.8).

Effects of notexin injection on force-generating capacity of old mdx mouse muscles

Having demonstrated that notexin pre-treatment could significantly increase AdV-mediated gene expression in old mdx muscles with advanced pathology, it was then important to evaluate the impact this intervention on muscle function. At 20 as well as 55 days after notexin injection, the specific force produced by the soleus muscle did not differ from control values across the force-frequency relationship (figures 4a & 5a). When specific force was expressed as a percent of the corresponding control muscle within the same animal to eliminate variance caused by biological variability of disease severity between animals, maximal twitch and tetanus values were similarly unaffected (figures 4b & 5b). Finally, absolute force production by notexin-treated muscles (i.e., without normalization for cross-sectional area) was not significantly different from contralateral control muscles at either time point (figures 4c & 5c). Regarding the kinetics of muscle contraction, notexin-treated muscles demonstrated a shorter contraction time and a trend toward shorter half relaxation time compared to saline injected muscle at the earlier time point (table 1).

Effects of combined notexin pre-treatment and AdV-Dys delivery on force-generating capacity of old mdx mouse muscles

In order to examine the functional effects of combined experimental regeneration and AdV-Dys gene transfer, soleus muscles were pre-treated with notexin and 5 days later injected with AdV-Dys. Figures 6a & b show typical examples of dystrophin immunostaining at 15 and 50 days after AdV-Dys injection, while group mean quantification of dystrophin positive myofibers is provided in figure 6c. There was no statistically significant difference in the number of dystrophin positive myofibers between the two time points (P=0.4).

At 15 days post-AdV-Dys delivery, there was no difference in specific force between treated and untreated soleus muscles across the force-frequency relationship, with the exception of a small but statistically significant increase at 10 Hz in the treated group (figure 7a). When values were normalized to corresponding control values within each individual animal, there was a significant improvement in specific twitch force in the notexin+AdV-Dys-treated group (figure 7b). However, as a consequence of a decline in soleus muscle cross-sectional area in the notexin+AdV-Dys-treated group at 15 days post-AdV-Dys (61 $\pm$ 5% of control; $P<0.05$), the absolute isometric force production by the muscle was not improved compared to the contralateral control muscle within the same animal (figure 7c).

At 50 days post-AdV-Dys delivery, the force-frequency relationship revealed an increase in specific force-generating capacity for muscles that received notexin+AdV-Dys at 10 Hz and 30 Hz, with a similar trend being observed at the higher stimulation frequencies (figure 8a). Along these same lines, specific force values normalized to the contralateral control muscle value showed increases of approximately 2-fold in maximal twitch force and the force generated at 10 Hz (figure 8b). Importantly, and in contrast to the data obtained at 15 days post-AdV-Dys, changes in the absolute isometric force generated by notexin+AdV-Dys-treated muscles at 50 days (figure 8c) paralleled the increases in specific force due to recovery of muscle cross-sectional area (94 $\pm$ 6% of control; not significantly different).

Effects of notexin+AdV-Dys administration on the ability to resist contraction-induced mechanical injury

It has been previously demonstrated that dystrophin deficient myofibers exhibit an increased susceptibility to contraction-induced mechanical injury, and this is reflected by an accentuated loss of force generating capacity following the application of lengthening (eccentric) contractions (32). Figure 9a depicts the

degree of force decline observed in muscles that received notexin+AdV-Dys 50 days earlier, as compared to the contralateral saline-injected muscle. There was increased resistance to contraction-induced mechanical injury (as indicated by a lesser loss of force-generating capacity) in the AdV-Dys-treated muscles. In addition, as shown in figure 9b, there was a significant relationship between protection from injury and the number of myofibers expressing dystrophin in the AdV-Dys-treated muscles.

Table 1: Short-term (20 days) and long-term (55 days) effect of notexin on muscle kinetics, Values represent means ± SE, * P< 0.05 versus the saline injected muscle.

Groups *(mice)*	n	Contraction Time *(ms)*	Half Relaxation Time *(ms)*
mdx *short-term*	9		
Saline		51.3±2.6	71.0±6.3
Notexin-treated		41.0±1.3*	53.0±5.0
mdx *(long-term)*	6		
Saline		49.0±2.2	72.0±10.0
Notexin-treated		47.0±2.7	68.0±13.0

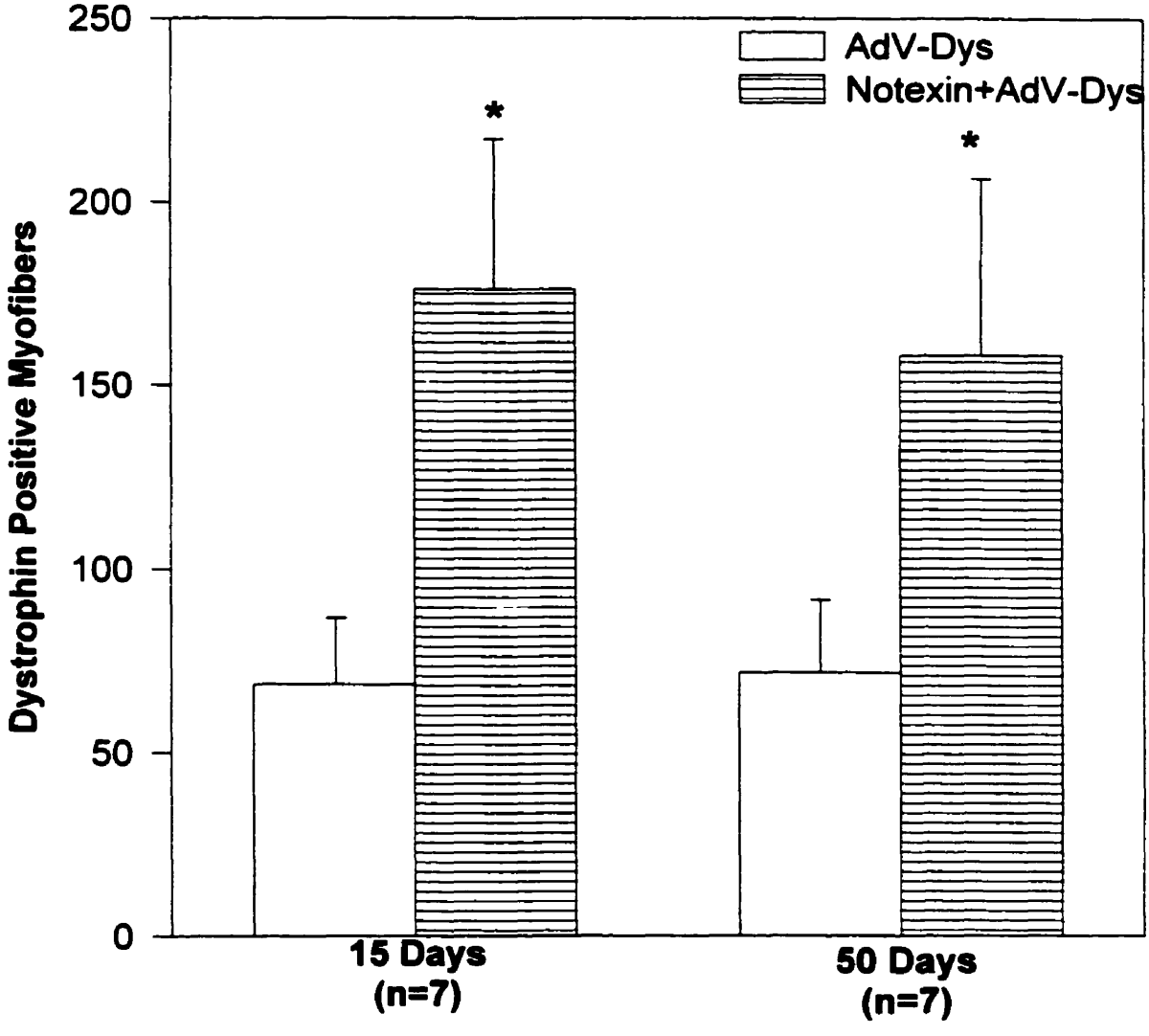
Table 2: Muscle kinetics 15 and 50 days after AdV-Dys delivery in notexinized muscles, Values represent mean ± SE

Groups *(mice)*	n	Contraction Time *(ms)*	Half Relaxation Time *(ms)*
mdx (15 days)	5		
Saline		51.3±2.6	57.0±5.9
Notexin-treated+AdV		44.0±1.4	71.0 ±6.6
mdx (50 days)			
Saline	5	44.2±2.1	59.0±6.0
Notexin-treated+ AdV		49.1±2.2	72.0±10.3

Figure 1. Efficiency of regeneration and upregulation of CAR after experimental induction of muscle regeneration with notexin in old mdx mice. Tibialis anterior (TA) muscles were injected with notexin or saline (negative control) and 5 days later reacted with antibodies against embryonic MHC and CAR. The negative control TA immunostained for embryonic MHC is shown in **(A)**, and a serial section reacted with anti-CAR antibody is shown in **(B)**. Expression of embryonic MHC and CAR in the negative control are minimal, and mostly present in the same small fibers (arrow). The notexin-treated TA immunostained for embryonic MHC **(C)** and CAR **(D)** shows widespread immunoreactivity and strong correspondence between embryonic MHC and CAR staining on serial sections (see orienting asterisk). Magnification: X 200.

Figure 2. Dystrophin immunostaining after AdV-Dys delivery to notexinized tibialis anterior muscle. The muscles were pre-treated with saline or notexin and 5 days later were injected with AdV-Dys. Examples are shown of the level of expression obtained in: **(A)** saline pre-treated TA 15 days after AdV-Dys delivery. Note the presence of cytoplasmic as well as sarcolemmal staining, indicating a high level of dystrophin transgene overexpression.. At 50 days the level of dystrophin expression in saline pre-treated TA was similar (data not shown). **(B)** Notexin pre-treated muscle 15 days after AdV-Dys delivery. **(C)** Notexin pre-treated muscle 50 days after AdV-Dys delivery. Magnification: X 200.

Figure 3. Quantitative evaluation of the number of dystrophin-expressing myofibers 15 and 50 days post-AdV-Dys injection in saline pre-treated and notexin pre-treated tibialis anterior muscle. There are significantly more dystrophin-expressing myofibers in notexinized muscles compared to saline pre-treated muscles at both 15 and 50 days after vector administration. Values represent mean values ±SE * $P < 0.05$ versus the saline pre-treated muscle.


250
200
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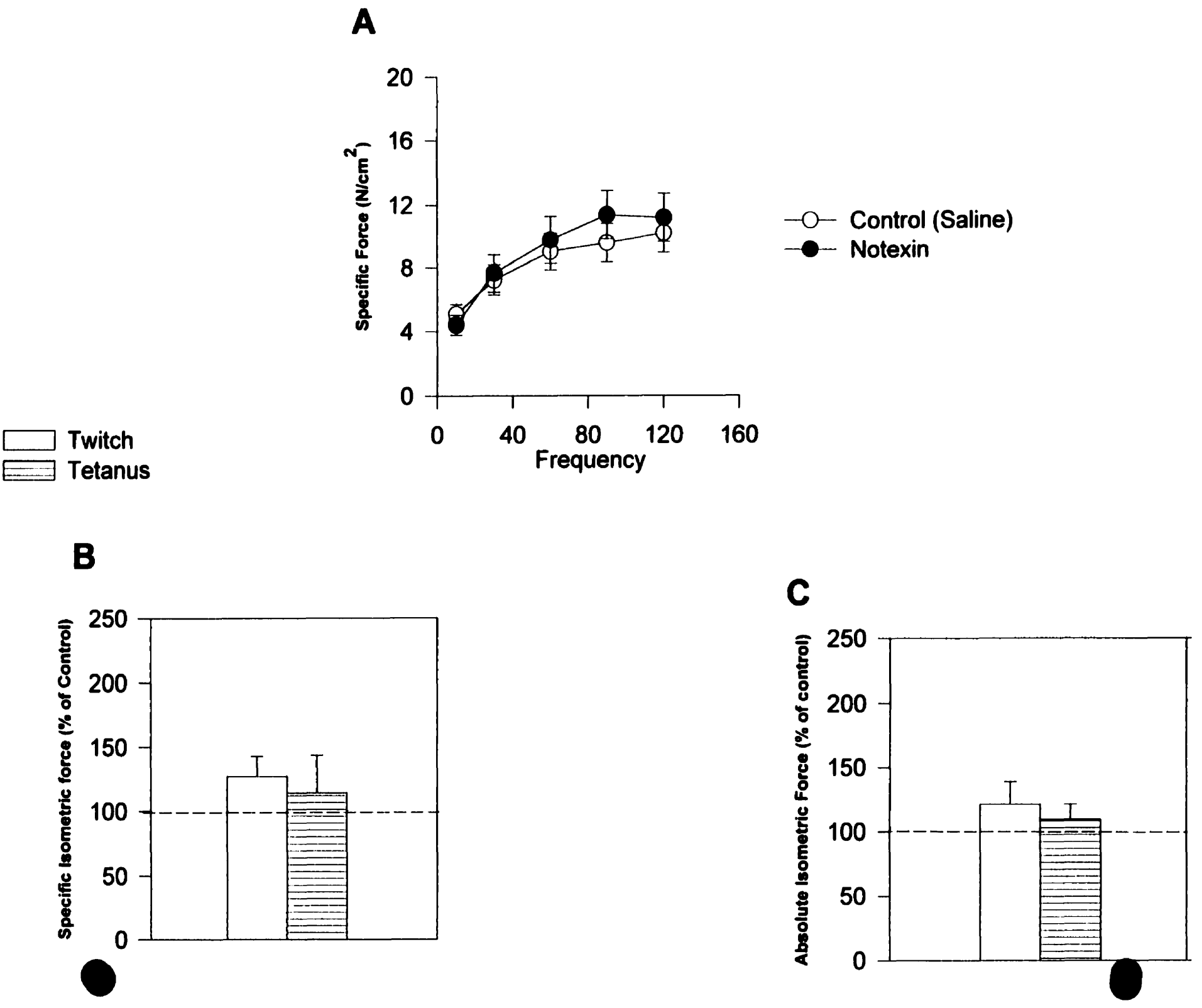
Dystrophin Positive Myofibers
AdV-Dys
Notexin+AdV-Dys
*
*
15 Days
(n=7)
50 Days
(n=7)

Figure 4. Force-generating capacity is not altered at short-term (20 days) after notexin-induced regeneration in old mdx soleus muscles. **(A)** Force-frequency relationship; **(B)** Maximal twitch and tetanic force normalized to muscle cross-sectional area; **(C)** Maximal values for absolute twitch and tetanic force. For (B) and (C), data are expressed as a percentage of the contralateral saline-injected soleus in the same animal All values represent means ±SE (n=9 per group).


A
20
16
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4
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Specific Force (N/cm2)
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80
120
160
Frequency
Control (Saline)
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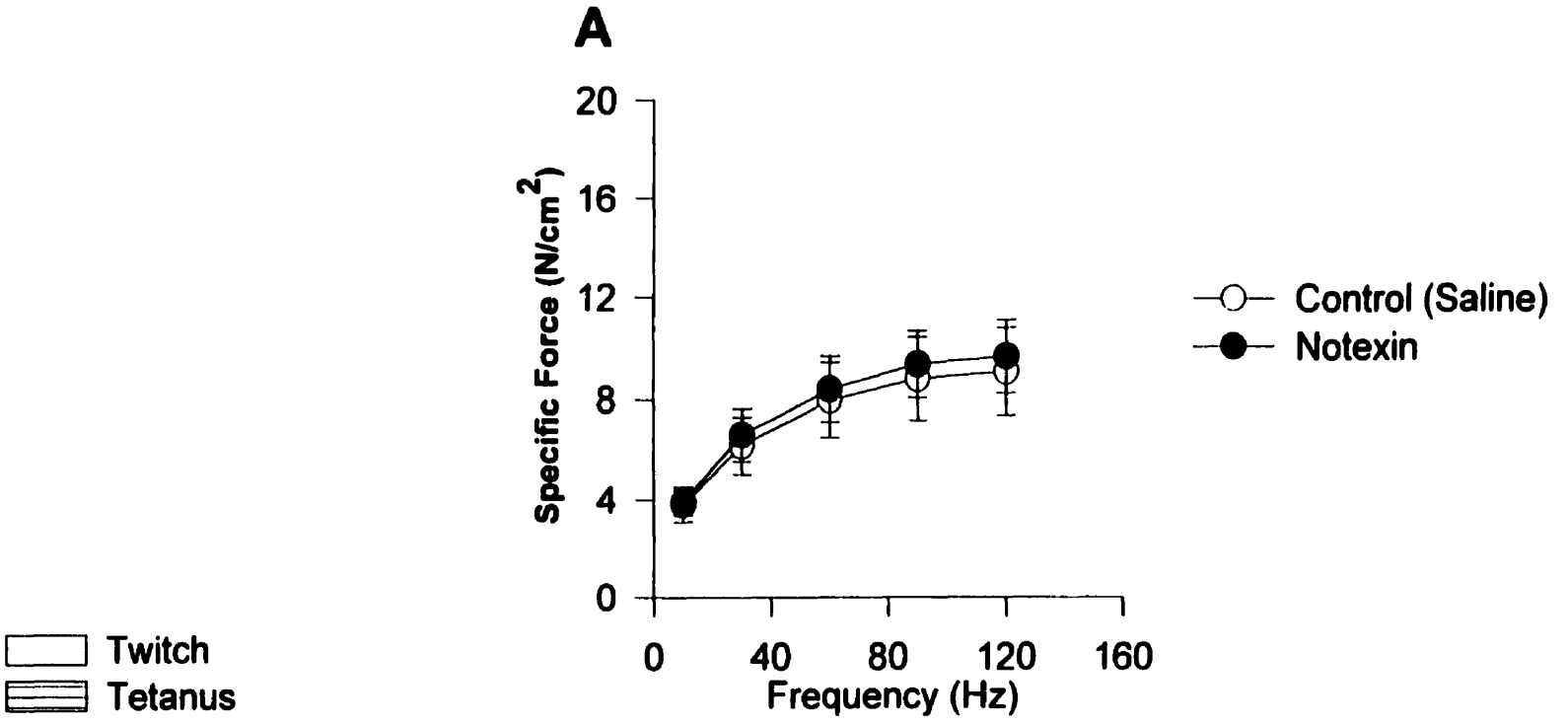
Twitch
Tetanus
B
250
200
150
100
50
0
Specific Isometric force (% of Control)
C
250
200
150
100
50
0
Absolute Isometric Force (% of control)

Figure 5. Force-generating capacity is maintained at longer term (50 days) after notexin-induced regeneration in old mdx soleus muscles. **(A)** Force-frequency relationship; **(B)** Maximal twitch and tetanic force normalized to muscle cross-sectional area; **(C)** Maximal values for absolute twitch and tetanic force. For (B) and (C), data are expressed as a percentage of the contralateral saline-injected soleus in the same animal All values represent means ±SE (n=6 per group).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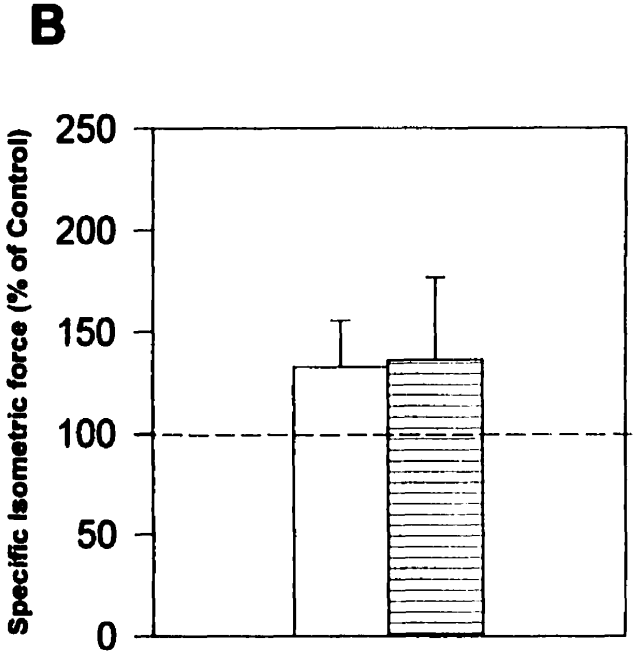
Frequency (Hz)
Control (Saline)
Notexin
Twitch
Tetanus


B
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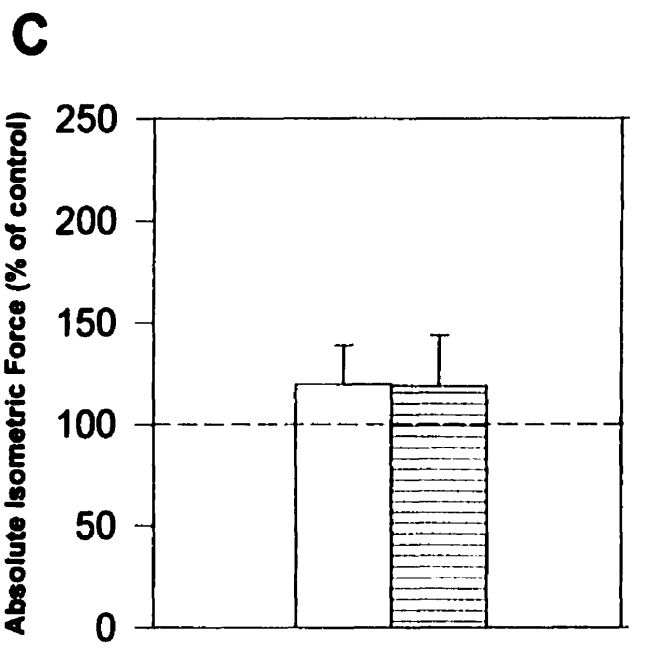
Specific Isometric force (% of Control)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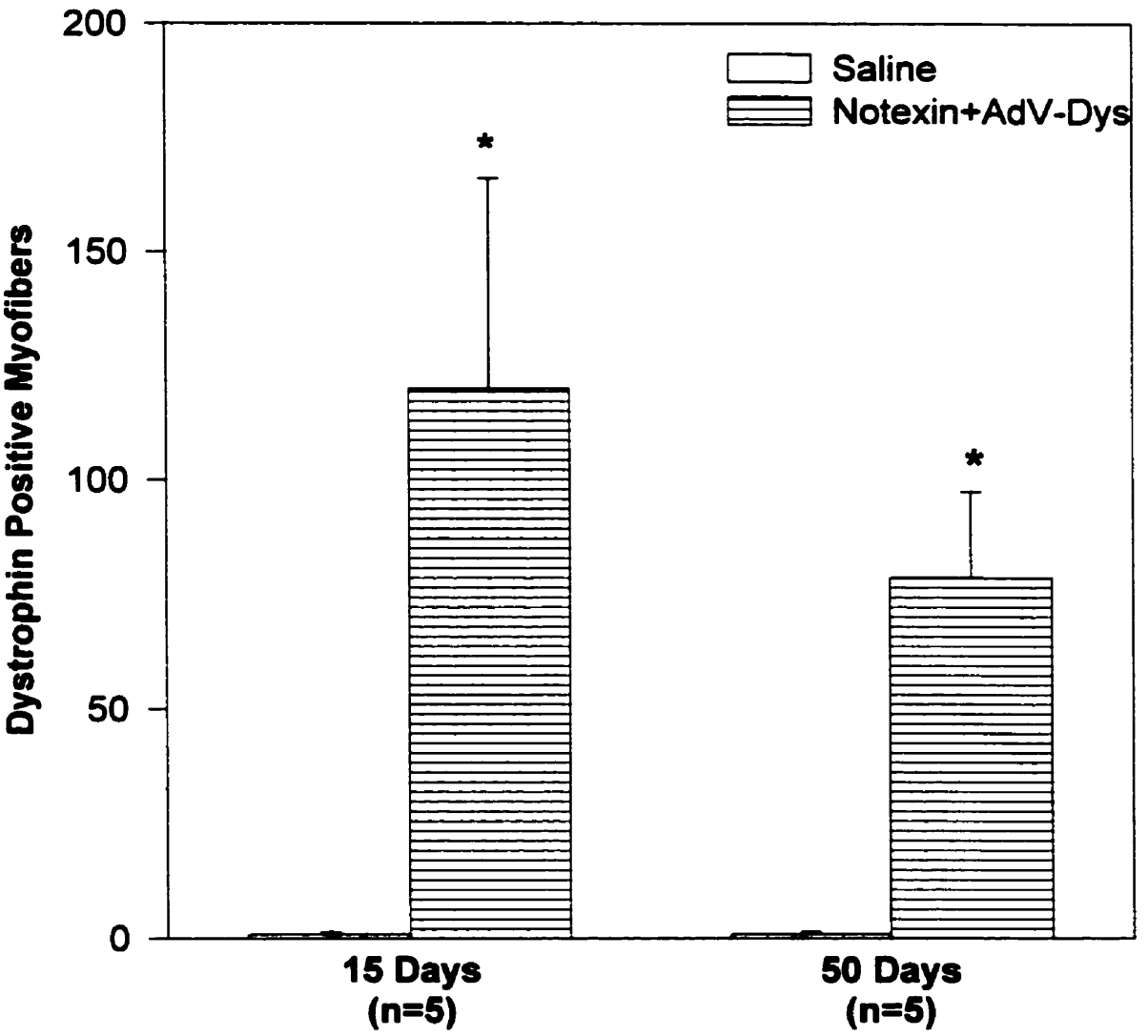
Absolute Isometric Force (% of control)

Figure 6. Dystrophin expression 15 days and 50 days after AdV-Dys injection into notexin pre-treated soleus muscles. **(A)** Example of the level of dystrophin-expressing myofibers 15 days after AdV-Dys delivery. **(B)** Example of the level of dystrophin-expressing myofibers 50 days after AdV-Dys delivery. Magnification: X 100. **(C)** Quantitative data for dystrophin-expressing myofibers at 15 and 50 days after AdV-Dys administration. There was no significant difference between 15 and 50 days in the number of dystrophin positive myofibers (P= 0.4). Values represent means ± SE (n=5).* P<0.05 versus the sham (saline)-treated muscle that did not receive AdV-Dys.

C


200
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Dystrophin Positive Myofibers
Saline
Notexin+AdV-Dys
*
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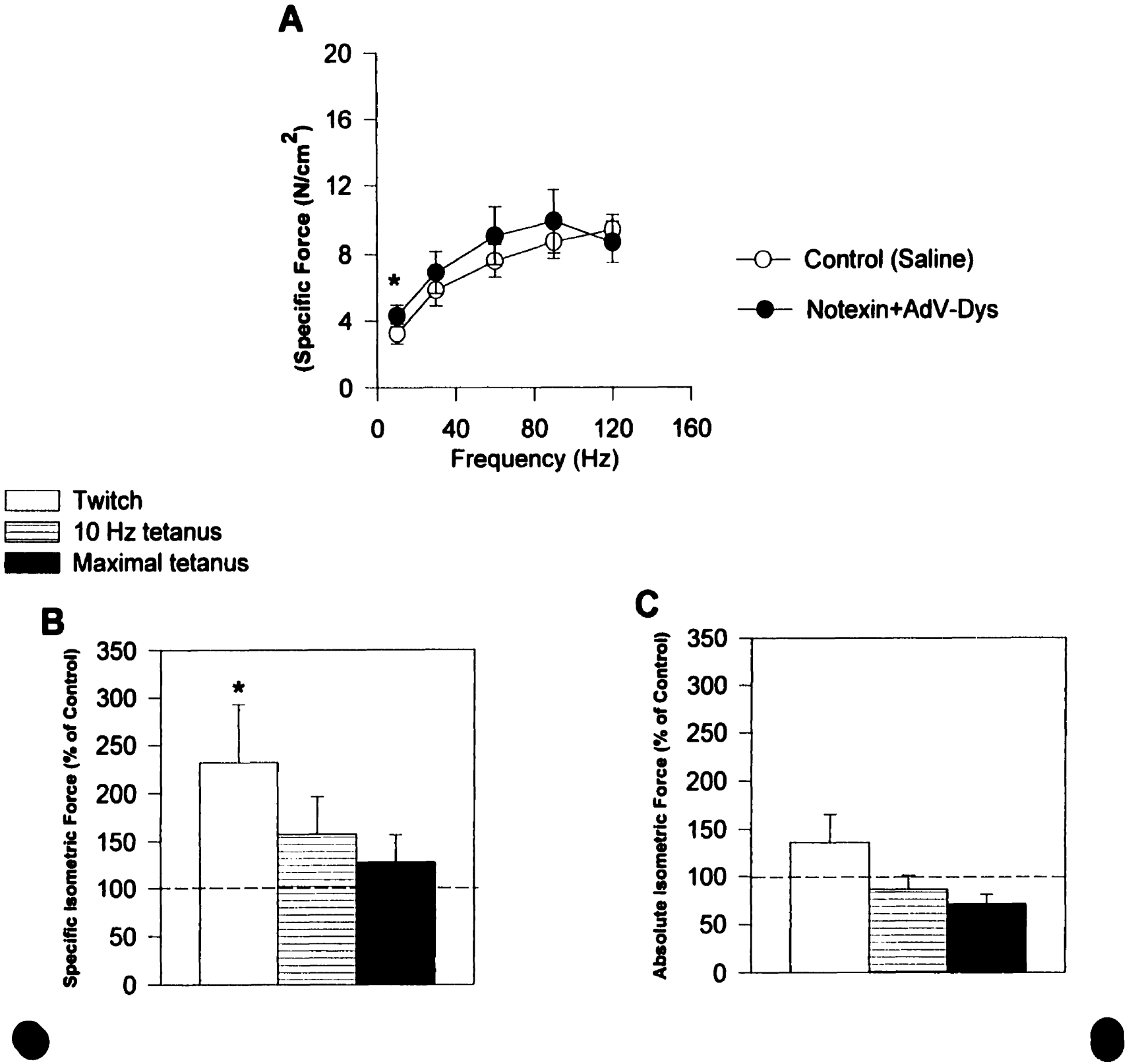
15 Days
(n=5)
50 Days
(n=5)

Figure 7. Effects of notexin and AdV-Dys on force-generating capacity 15 days after AdV-Dys delivery. **(A)** Force-frequency relationship; **(B)** Maximal twitch and tetanic force normalized to muscle cross-sectional area; **(C)** Maximal values for absolute twitch and tetanic force. For (B) and (C), data are expressed as a percentage of the contralateral saline-injected soleus in the same animal All values represent means ±SE (n=5 per group). * $P<0.05$ versus the sham (saline)-treated muscle that did not receive AdV-Dys.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(Specific Force (N/cm2)
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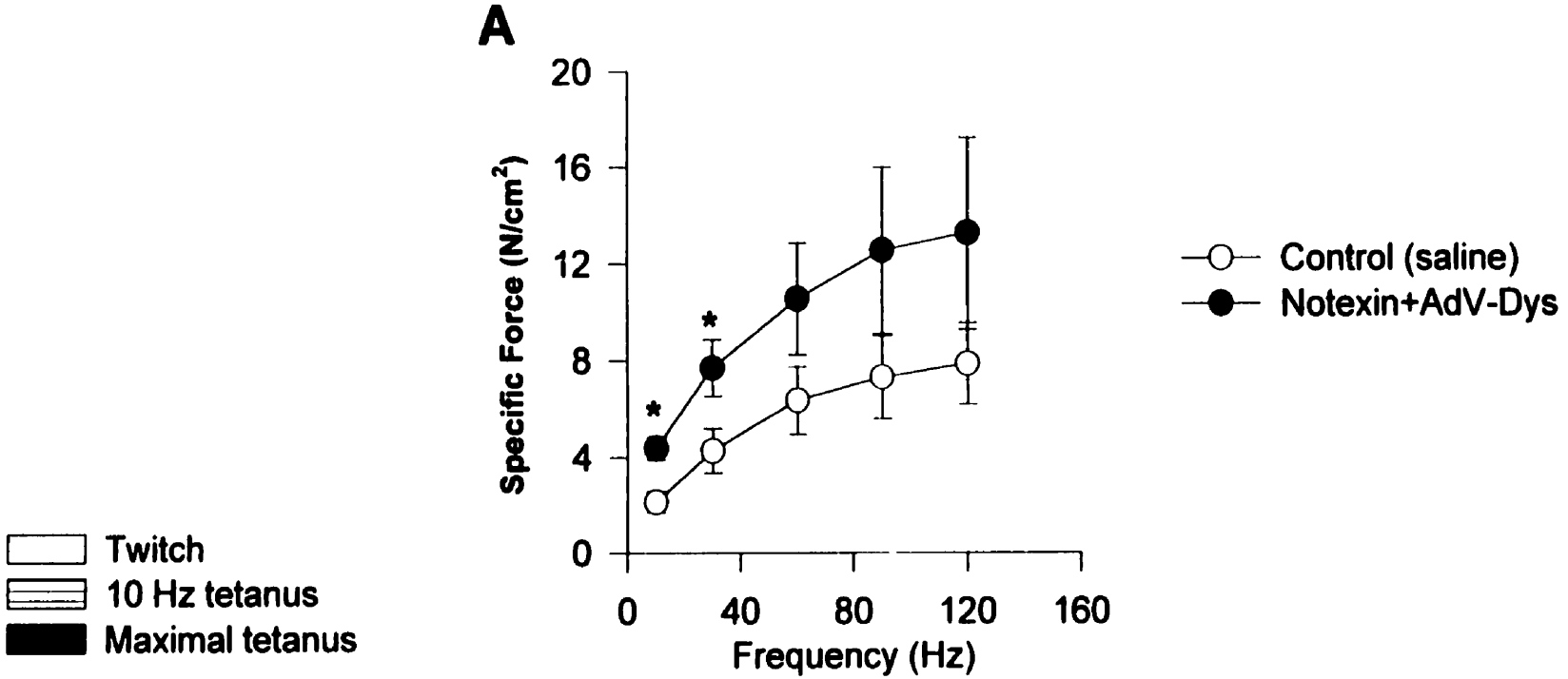
Frequency (Hz)
Control (Saline)
Notexin+AdV-Dys
Twitch
10 Hz tetanus
Maximal tetanus
B
Specific Isometric Force (% of Control)
350
300
250
200
150
100
50
0
*
C
Absolute Isometric Force (% of Control)
350
300
250
200
150
100
50
0

Figure 8. Effects of notexin and AdV-Dys on force-generating capacity 50 days after AdV-Dys delivery. **(A)** Force-frequency relationship; **(B)** Maximal twitch and tetanic force normalized to muscle cross-sectional area; **(C)** Maximal values for absolute twitch and tetanic force. For (B) and (C), data are expressed as a percentage of the contralateral saline-injected soleus in the same animal. Note the increase of force-generating capacity in the notexin+AdV-Dys-treated soleus compared to control at lower stimulation frequencies. All values represent means ±SE (n=5 per group). * $P<0.05$ versus the sham (saline)-treated muscle that did not receive AdV-Dys.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Specific Force (N/cm²)
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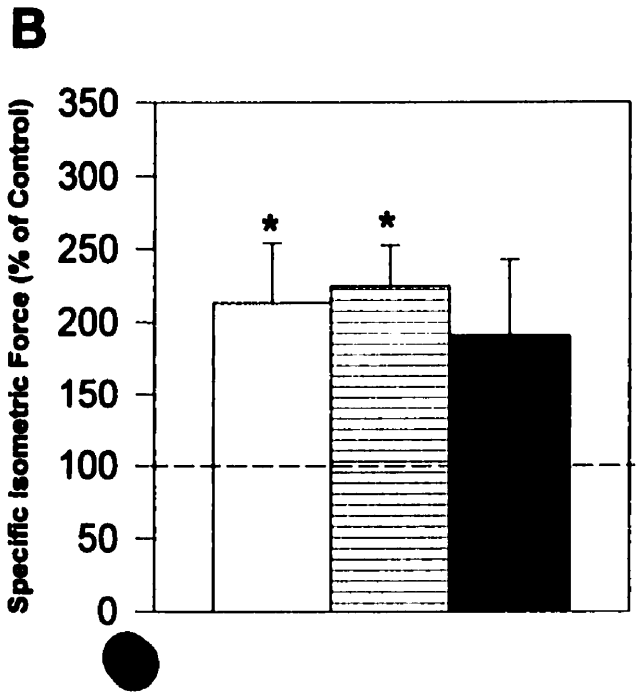
Frequency (Hz)
Control (saline)
Notexin+AdV-Dys
Twitch
10 Hz tetanus
Maximal tetanus


B
Specific Isometric Force (% of Control)
350
300
250
200
150
100
50
0

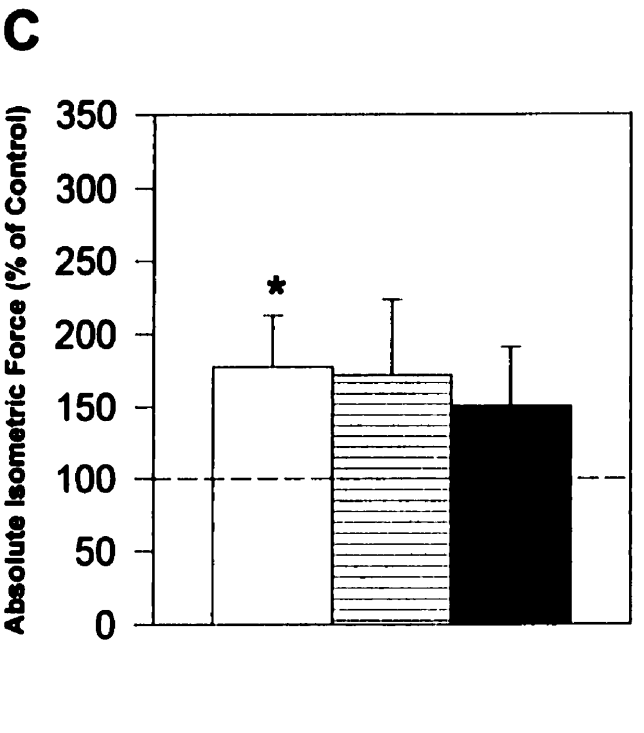
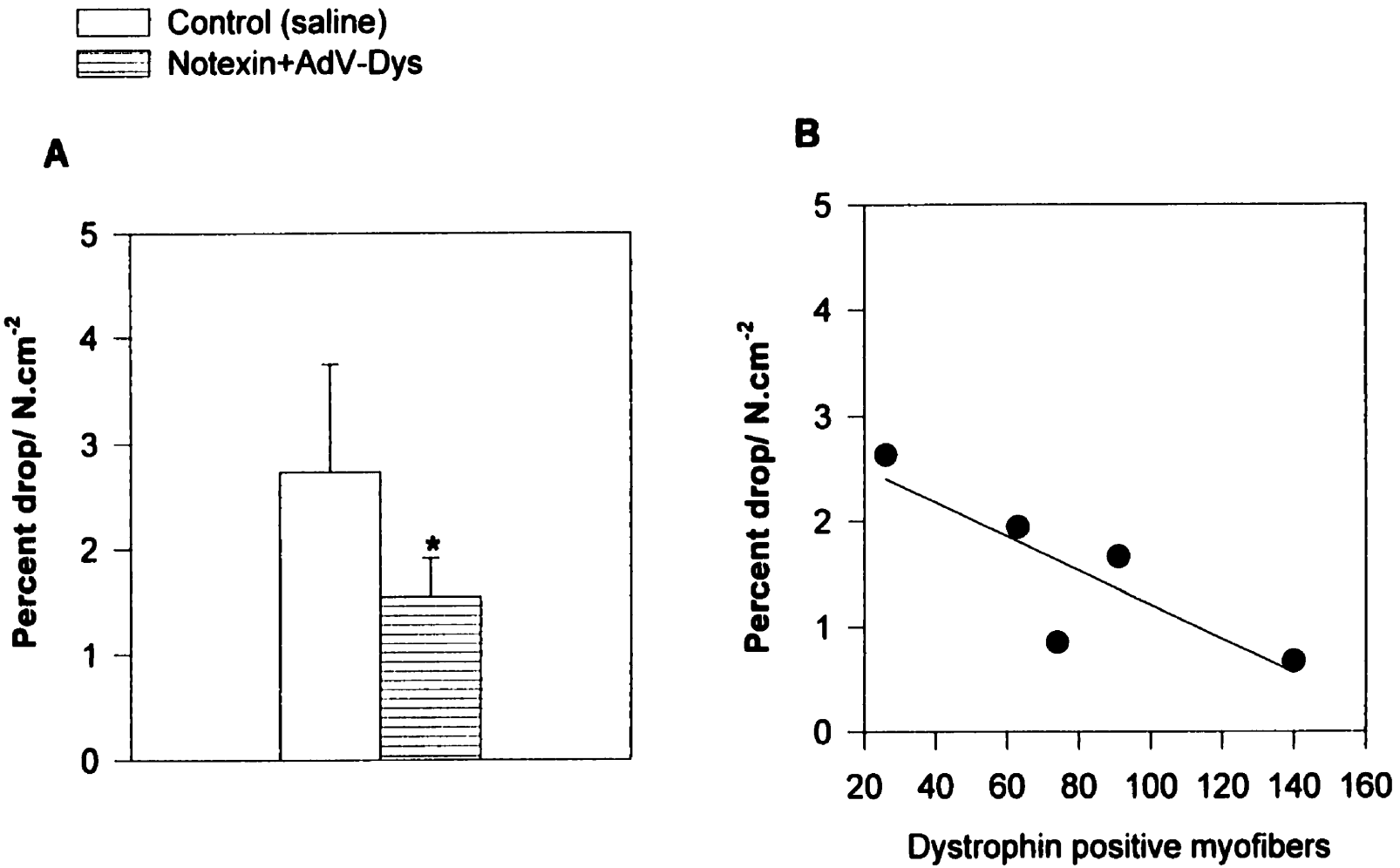

C
Absolute Isometric Force (% of Control)
350
300
250
200
150
100
50
0

Figure 9. Force decline in response to high-stress lengthening (eccentric) contractions at 50 days after AdV-Dys administration. **(A)** Eccentric contractions produced a lesser degree of force decline in notexin+AdV-Dys-treated soleus muscles as compared to control saline-treated muscles ($P<0.05$). Values represent means ± SE and are expressed as percent decline of force normalized to the maximal isometric stress imposed (n=5). **(B)** Correlation between eccentric contraction-induced force decline and the number of dystrophin-expressing myofibers ($R^2=0.70$, $P<0.05$).


Control (saline)
Notexin+AdV-Dys
A
Percent drop/ N.cm-2
0
1
2
3
4
5
*
B
Percent drop/ N.cm-2
0
1
2
3
4
5
20
40
60
80
100
120
140
160
Dystrophin positive myofibers

3.6 Discussion

The major findings of the present study can be summarized as follows: 1) notexin administration to old mdx muscles led to effective CAR upregulation and a correspondingly increased efficiency of AdV-mediated dystrophin gene transfer; 2) notexin injection did not produce adverse effects on muscle force-generating capacity at the time points examined, but when combined with AdV-Dys administration caused a transient decrease in muscle cross-sectional area that subsequently recovered to baseline levels by approximately 2 months post-treatment; and 3) combinatorial treatment with notexin followed by AdV-Dys was able to improve maximal twitch as well as tetanic force generation at the lower range of muscle stimulation frequencies despite the presence of pre-existent advanced pathology in old mdx mouse muscles.

Current evidence suggests that myofibers lacking dystrophin are abnormally susceptible to contraction-induced damage (32;37), which secondarily leads to muscle fiber dysfunction (12;38), necrosis and eventual replacement of the lost fibers by connective tissue. Therefore, the muscle weakness found in DMD patients is due to both myofiber loss and impaired contractile function in the surviving myofiber population (12). Gene therapy approaches in DMD should ideally be capable of reversing and/or preventing these changes without entailing significant adverse effects on muscle function. Previous studies examining the ability of AdV-mediated dystrophin gene transfer to improve muscle force-generating capacity have been limited to neonatal or young adult mdx mice (9;39). Here we show for the first time that in old mdx muscles with more severe and established pathology, AdV-Dys administration preceded by notexin-induced "priming" of the muscle is capable of improving isometric force production at the lower stimulation frequencies that are most characteristic of normal everyday muscle use. In addition, this strategy led to an increase in the ability of dystrophic myofibers to resist contraction-induced injury, the latter having been implicated as an important element in dystrophin deficiency disease

pathogenesis (32;37) as noted above. This occurred in a manner that was significantly correlated with the number of dystrophin-expressing fibers, strongly suggesting that the effect was due to the presence of the therapeutic gene.

The efficiency of AdV-mediated gene transfer has been shown to be markedly reduced in mature as compared to immature muscle fibers (1;11;17). Although muscles of young mdx mice show a substantial number of immature and recently regenerated myofibers, older mdx mice contain relatively few (10) and are also less easily transduced by AdV (2). In the present study, we employed mdx mice that had attained about 70% of their expected lifespan (30), an age at which a decreased myogenic capacity (4;30;41) and increased muscle fibrosis (28;35) have been documented. It is important to note that this situation closely resembles the usual clinical scenario at the time of diagnosis in DMD patients (6;36). There is evidence that the reduced ability of AdV to infect mature myofibers is due at least in part to decreased expression of the high-affinity primary attachment receptor for adenovirus, known as CAR (5;29). The fact that notexin pre-treatment was able to acutely increase CAR expression in this context, without incurring significant long-term adverse effects on muscle function, suggests that these muscles are still able to respond adequately to externally imposed cycles of necrosis and regeneration.

It is nonetheless important to recognize that although notexin alone did not adversely affect muscle function in our study, the serial delivery of notexin and AdV-Dys caused a transient reduction of muscle mass and cross-sectional area. We speculate that at least two factors could delay the steps of myoblast proliferation, fusion and differentiation needed to reconstitute the muscle after experimentally-induced necrosis. First, there could be an augmented release of catabolic cytokines by inflammatory cells triggered in response to the superimposed delivery of AdV-Dys to the muscle. However, to the extent that increased levels of CAR on regenerating fibers may reduce the local muscle

concentration of AdV particles required for effective gene delivery, the use of notexin may actually allow one to decrease the initial AdV dosage and thus mitigate this problem to a considerable degree. Second, there may direct interference with cellular functions involved in muscle regeneration, including muscle cell protein synthesis (15), by leaky expression of adenoviral gene products (8;40). This problem should be avoidable through the use of the latest generation of adenoviral vectors (so-called gutless AdV) that have been fully deleted of all viral coding sequences (14;20).

Finally, it should be noted that in addition to CAR upregulation, the beneficial effects of notexin pre-treatment on the subsequent efficiency of AdV-mediated gene expression could be exerted through multiple pathways. Thus, not only CAR but also the lower affinity internalization receptors for adenovirus, are relatively upregulated in immature muscle (1). In addition, notexin may have effects on the extracellular matrix which favor vector distribution and uptake by muscle cells (19). It is also conceivable that an inherently greater transcriptional and/or translational activity of immature myofibers could contribute to the higher levels of transferred gene expression observed after AdV-Dys delivery to notexinized muscles.

In summary, the risk:benefit ratio associated with the induction of a single cycle of necrosis-regeneration in old mdx muscles as a method for increasing the efficiency of subsequent AdV-mediated dystrophin gene transfer, appears to be favorable. Conceptually, this approach is in many respects analogous to autologous bone marrow transplantation, in which diseased cells are initially destroyed and the organ is then reconstituted with genetically modified cells from the same host. To the extent that the genetically modified cells are able to stably express the therapeutic gene product, the need for experimental induction of necrosis should be a one-time event. The question of whether a similar

paradigm can be employed in DMD patients to increase the efficacy of AdV-mediated dystrophin gene transfer deserves further investigation.

3.7 References

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Chapter 4

Combinatorial blockade of calcineurin and CD28 signaling facilitates primary and secondary therapeutic gene transfer by adenovirus vectors in dystrophic (mdx) mouse muscles

4.1 Prologue

In the first two chapters of this thesis we have modified the host by experimentally inducing muscle regeneration to force the expression of AdV-permissive immature myofibers. Thus, the preceding experimental work constituted only one part of host modification strategies that we had intended to perform. In the following chapter, we will discuss of an immunosupressive approach that has allowed us to tackle the immune response that is generated after AdV-Dys delivery. We report that by interfering with key steps leading to an optimal immune reaction, one can abrogate partially or almost entirely immune-mediated loss of AdV-Dys transduced myofibers. The overall modality of immunosupression has consisted at blocking:

1) The adhesion between antigen presenting cells (APC) and T cells through the use of anti CD2, anti ICAM-1 and anti LFA-1 immunomodulatory antibodies.

2) The recognition of the foreign antigen presented to T cell receptors (TCR) by administrating FK506 drug which blocks through inactivation of calcineurin TCR signaling events.

3) The costimulation of the T cell (through CD28 and accessory molecules expressed on APC [B7-1 or B7-2]) by administrating a fusion molecule CTLA4Ig which possesses higher avidity for B7 molecules comparatively to CD28.

Therefore, based upon the examination of the individual effects of each of these immunomodulatory regimens on muscle inflammation following AdV-Dys delivery, we have explored the possibility of the existence of synergistic actions between the inhibition of TCR signaling events and the blockade of CD28-mediated costimulation pathway. Thus, we report that the later-mentioned synergistic approach abrogates the

immune response against the adenovirus proteins and dystrophin transgene following to the AdV-Dys transfer in mdx dystrophic mice.

4.2 Abstract

Recombinant adenovirus vectors (AdV) have been considered as a potential vehicle for performing gene therapy in patients suffering from Duchenne muscular dystrophy, but are limited by a cellular and humoral immune response that prevents long-term transgene expression as well as effective transduction after AdV readministration. Conventional immunosuppressive agents such as cyclosporine and FK506, which act by interfering with CD3-T cell receptor-mediated signaling via calcineurin, are only partially effective in reversing these phenomena and may also produce substantial organ toxicity. We hypothesized that activation of redundant T cell activation pathways could limit the effectiveness of these drugs at clinically tolerable doses. Therefore, we have tested the ability of immunomodulatory immunoglobulins (Ig) with different modes of action to facilitate AdV-mediated gene transfer to adult dystrophic (mdx) mice. When used in isolation, immunomodulatory Ig (anti-ICAM-1, anti-LFA-1, anti-CD2, CTLA4Ig) were only mildly effective in mitigating cellular and/or humoral immunity against adenoviral capsid proteins and the therapeutic transgene product, dystrophin. However, the combination of FK506+CTLA4Ig abrogated the immune response against adenoviral proteins and dystrophin to a degree not achievable with the use of either agent alone. At 30 days after AdV injection, >90% of myofibers could be found to express dystrophin with little or no evidence of a cellular immune response against transduced fibers. In addition, the humoral immune response was markedly suppressed, and this was associated with increased transduction efficiency following vector readministration. These data suggest that by facilitating both primary and secondary transduction after AdV administration, combined targeting of CD3-T cell receptor-mediated signaling via calcineurin and the B7:CD28 costimulatory pathway could greatly increase the potential utility of AdV-mediated gene transfer as a therapeutic modality for genetic diseases such as Duchenne dystrophy that will require long-term transgene expression and repeated vector delivery.

4.3 Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic and ultimately fatal disorder that afflicts approximately 1 in 3500 male newborns. The primary defect is an absence of dystrophin (17), a subsarcolemmal protein believed to play an important role in providing structural reinforcement to the muscle cell surface membrane (31;38). First generation adenovirus vectors (AdV), which have been made replication-defective by deleting early region 1 (E1) from the vector genome, have been used to achieve AdV-mediated transfer of a 6.3 kb dystrophin minigene in vivo (39),(2) (20;33). AdV infect non-replicating cells such as skeletal muscle fibers with a relatively high degree of efficiency (1;2), and it has recently been reported that AdV-mediated dystrophin minigene transfer is capable of ameliorating muscle function in an animal model of DMD, the mdx mouse (8;36). However, in order for this beneficial effect to be realized, animals must be either immunologically immature (8) or actively immunosuppressed with potent drug therapy (36). In the presence of an intact immune system, CD8+ cytotoxic T lymphocytes (CTLs) destroy the AdV-infected myofiber population (2;37;44) and also produce an accompanying worsening of muscle contractile function (35;37).

Although substantial progress has been made in developing less immunogenic vectors through the inactivation (47) or deletion of viral genome elements (12;16), (5) this approach has at least two inherent limitations with respect to the treatment of monogeneic recessive disorders such as DMD. Firstly, since AdV particle neutralization by antibodies directed against inoculum capsid proteins is believed to be the principal mechanism preventing effective readministration of AdV (23;46), it is doubtful that this problem can be overcome by further modification of the vector genome. Secondly, the therapeutic transgene protein product would itself represent a neoantigen that could, depending upon its own intrinsic immunogenicity, stimulate host cellular immunity with attendant elimination of AdV-infected cells. Indeed, the magnitude and nature of host immune responses to foreign gene transfer appears to vary considerably depending

upon the specific transgene product being expressed (7) (32). For this reason, it is exceedingly important that proposed immunosuppressive regimens be tested not only with non-therapeutic marker genes as has been the case in many prior studies (14;21), (35;37;44;48), but also with the specific therapeutic transgene of clinical interest.

Based upon the above considerations, the development of safe and effective methods for downregulating the host immune response against both adenoviral capsid proteins and dystrophin is a likely prerequisite to the eventual application of any type of AdV-mediated gene transfer in DMD patients. Distinct stages of cell-cell interaction between antigen-presenting cells (APCs) and T cells are normally involved in the induction of an antigen-specific immune response (see ref (15) for review). These include: 1) adhesion between APC and T cell, 2) recognition of foreign antigen presented to T cell receptors located in the CD3 complex on the T cell surface, and 3) costimulation of the T cell by accessory molecules present on the APC, which triggers subsequent T cell proliferation and effector function. Commonly employed immunosuppressive drugs such as cyclosporine and FK506 exert their effects by blocking T cell signaling events associated with the CD3-T cell receptor pathway, thereby inhibiting IL-2 production (11;22;28). We have previously reported that FK506, which blocks T cell signaling by calcineurin, a Ca^{2+}- and calmodulin-dependent phosphatase (28), significantly increased the level of dystrophin gene expression after a single delivery of AdV to muscles of mdx mice (29). However, FK506 was only partially effective in blocking the generation of antibodies against adenoviral capsid proteins and permitting further dystrophin gene expression after a second AdV injection (29). Although this problem might theoretically be overcome through the use of higher drug doses, in clinical practice this approach is often limited by substantial organ toxicity as well as an increased risk of host infection. Furthermore, even in the presence of maximally tolerated doses of FK506 or related compounds, T cell activation could potentially occur via redundant signaling pathways that are unaffected by blockade of CD3-T cell receptor-mediated lymphocyte activation

(11;22). In this regard, it is particularly noteworthy that T lymphocyte activation induced by the interaction between B7-1 (CD80) or B7-2 (CD86) accessory molecules on APCs and CD28 molecules present on T cells, which constitutes perhaps the most important costimulation pathway (15) (9), is distinct from the CD3-T cell receptor signaling pathway and therefore not inhibited by either cyclosporine or FK506(22) (11).

Adhesion molecule pairings between intercellular adhesion molecule (ICAM)-1 and leukocyte function-associated antigen (LFA)-1, as well as between LFA-3 and CD2, have been shown to be important in facilitating foreign antigen recognition by T lymphocytes in vivo (4;13;19). Whereas the former interaction appears to be largely dependent upon the presence of T cell activation, the latter is reported to be essentially independent of this parameter, thus suggesting the possibility of differential roles for these adhesion pairs (34). In addition, the fusion protein CTLA4Ig (27), which has a higher avidity for B7 molecules than CD28 and an inhibitory effect on CD28-mediated T cell activation (9;15;27;42), has been shown to produce organ allograft acceptance in animal models (15;25;26) as well as persistent transgene expression after liver-directed AdV-mediated gene transfer (23). Therefore, in the present study we have employed immunomodulatory immunoglobulins (Ig) to impede these specific adhesion and costimulatory molecule interactions, in order to determine whether short-term interference with receptor-ligand pairings normally involved in T cell activation enhances the efficacy of AdV-mediated dystrophin gene transfer in adult dystrophic (mdx) mice. Furthermore, we have attempted to ascertain the existence of any additive or synergistic effects when using a combinatorial strategy to inhibit both CD3-T cell receptor-mediated signaling via calcineurin and the CD28-mediated costimulatory pathway. Here we report that the latter approach in particular markedly abrogates the immune response against adenoviral proteins and the dystrophin transgene product for primary as well as secondary AdV-mediated dystrophin gene transfer, thereby expanding the potential utility of this modality as a therapeutic option for DMD.

4.4 Materials and Methods

Preparation of Recombinant Adenoviruses

Adenoviral recombinants containing the 6.3 kb human dystrophin minigene (AdV-Dys) were constructed using E1/E3-deleted replication-defective serotype 5 human adenovirus as previously outlined in detail (2;37), where the transgene cDNA was driven by cytomegalovirus (CMV) promoter/enhancer elements inserted into the E1 region. The absence of contamination by E1-containing replication-competent AdV was confirmed using a sensitive PCR screening assay as previously described (30). AdV titers were determined by spectrophotometry at 260 nm and are expressed as particles/ml (2;37).

Immunomodulatory Reagents

Rat IgG directed against murine adhesion molecules ICAM-1 (anti-CD54, hybridoma YN1/1.7; American Type Culture Collection, Rockville, MD) and LFA-1 (anti-CD11a, hybridoma M17/4; American Type Culture Collection, Rockville, MD) were purified over protein G from hybridoma supernatant. Based upon a regimen previously described for cardiac allograft preservation in mice (19), AdV-injected mdx mice were treated with 100 mg of each monoclonal antibody by intraperitoneal (i.p.) injection daily, beginning the day of AdV-Dys administration and continuing for a total of 6 days. Rat IgG directed against murine CD2 (4) (13) (hybridoma 12-15, gift of P. Altevogt, Heidelberg, Germany) and an irrelevant (control) rat IgG were similarly purified and injected i.p. using the same dosing regimen. Human CTLA4Ig (gift of P. Linsley, Bristol-Meyers Squibb, Seattle, WA) is a soluble fusion protein containing the extracellular domain of the CTLA4 receptor together with the Fc domain of IgG, which inhibits T cell signaling via the B7:CD28 costimulation pathway (27) (15) (9). Using a dosing regimen previously reported to prolong transgene expression in mice for several months after AdV-mediated gene transfer to liver (23), CTLA4Ig was administered i.p. at a dose of 200 mg on days 0, 2 and 10 after AdV-Dys injection of mdx muscles. For mice treated

with FK506 (5 mg/kg/d subcutaneously), this immunosuppressive regimen was selected based upon our prior demonstration of sustained dystrophin expression 2 months after AdV-Dys delivery to mdx mice (29); the drug was begun on the day prior to AdV-Dys injection and continued until the animals were euthanized.

Animal Procedures and Experimental Protocols

Dystrophin-deficient mdx mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and entered into the study at 30-50 days of age. Prior to AdV injection, the mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg) by intramuscular (i.m.) injection into muscles other than those used for AdV-Dys injection. Target muscles were then surgically exposed to permit AdV-Dys injection under direct visualization. At the end of the designated experimental period, mice were euthanized by anesthetic overdose. All animal procedures were approved by the institutional animal ethics committee.

Use of Different Immunomodulatory Ig in Isolation. Mdx mice received 20 ml of purified AdV-Dys ($7x10^{11}$ particles/ml) into the anterior tibialis muscle. The animals were treated with one of the following: 1) Anti-ICAM-1/LFA-1, 2) Anti-CD2, 3) CTLA4Ig, or 4) Control rat IgG as described above. At 30 days after AdV-Dys administration, injected muscles were excised and frozen for immunohistochemistry; sera were also collected for detection of antibodies against adenoviral proteins and dystrophin (see below).

Use of Combinatorial Approach to Block Calcineurin and CD28 Costimulation Pathways. Animals were divided into 3 groups: 1) CTLA4Ig alone, 2) FK506 alone, and 3) FK506+CTLA4Ig. The dosing regimens for both CTLA4Ig and FK506 were as described above. Each group again received AdV-Dys into the right anterior tibialis muscle on day 0 (first administration), followed by the same dosage of AdV-Dys

delivered to the left anterior tibialis on day 20 (second administration); this sequence was selected to allow direct comparison to our prior study of FK506 therapy in the setting of AdV-mediated dystrophin gene transfer (29). All animals were subsequently euthanized on day 30, and the AdV-Dys-injected muscles as well as sera were collected.

Dystrophin Immunostaining and Quantitation of Inflammatory Response

Excised muscles were embedded in mounting medium and snap-frozen in isopentane pre-cooled with liquid N_2. Transverse cryostat sections (6 μm thick) were obtained from the midportion of the muscle and then fixed on slides in 1% acetone. Immunohistochemical procedures were carried out to detect dystrophin expression using a polyclonal antidystrophin (C-terminus) primary antibody and biotinylated secondary antibody with subsequent visualization by peroxidase staining, as previously outlined in detail (2) (29). Muscle sections were also counterstained with hematoxylin/eosin (H/E) to allow detection of inflammatory cell infiltration within AdV-injected muscles.

Microscopically visualized sections were photographed by video camera (magnification 100x) and the image was captured to a Macintosh computer using a frame-grabber. Analysis of the number of dystrophin-positive myofibers on the entire muscle cross-section was performed using the public domain program NIH Image (version 1.49). In order to quantify the magnitude of inflammation in AdV-Dys-injected muscles, standard point-counting technique was employed and the area fraction of inflammation was then determined as previously described (6). Briefly, 3-4 randomly selected microscopic fields per muscle were selected, and a 100-point grid was superimposed onto each captured image using a stereology software package (Stereology Toolbox, Morphometrix, Davis, CA). An abnormal point was defined as either falling upon inflammatory cells or a myofiber invaded by such cells. The area fraction of inflammation was calculated by dividing the number of abnormal points by the total

number of points falling on the tissue section, and is expressed as a percentage.

Measurement of Humoral Immune Responses

The host antibody response to adenovirus capsid proteins was measured by ELISA as previously described (37). Briefly, Nunc Maxisorb microtiter plates (GIBCO, Gaithersburg, MD) were coated with heat-inactivated AdV particles (1 x 10^8/well) overnight in 100 ml of sterile PBS (pH 7.2). Serum obtained from each individual mouse was then diluted in ELISA buffer (0.5% BSA, 0.05% Tween 20 in PBS), applied to the microtiter plate wells, and incubated overnight at 4EC. Reactivity to AdV was determined by incubation with horseradish peroxidase conjugates of goat anti-mouse IgG (1:1000; Serotec, Toronto, Ontario) for 1 hour, followed by a washing step and the addition of enzyme substrate (100 ml/well of 0.1 mg/ml of 2,2'-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium in 0.1 M citrate buffer, pH 4.5) and 0.01% H_2O_2. Absorbance was read at 405 nm on an Easy Reader-400AT (SLT Lab Instruments, Salzburg, Austria). Sera from naive non-AdV-injected mdx mice served to establish background absorbance values, and data from all experimental groups are expressed as a percent of the naive serum value.

The humoral immune response to human dystrophin in AdV-Dys-injected animals was assessed using a previously described immunocytochemical detection system (29). Human muscle biopsy specimens were obtained from individuals without histological evidence of neuromuscular disease. Mouse sera from each AdV-Dys-injected experimental group were pooled, while sera from naive non-AdV-injected mdx mice served as a negative control. Sections of normal human skeletal muscle were then blocked with 10% goat serum in PBS for 1 hour, followed by incubation with serial dilutions of pooled mouse sera (up to a maximum dilution of 1:70000) in blocking buffer overnight. Secondary antibody (1:200) consisted of a biotinylated antimouse IgG raised in horse (Vector, Burlingame. CA, USA), which was applied for 1 hour. Sections were

then reacted with Cy3-conjugated streptavidin (1:1000) for 20 min (Jackson ImmunoResearch, West Grove, PA, USA), mounted, and viewed under epifluorescence microscopy. Mouse sera which generated visually detectable sarcolemmal staining on human muscle sections were considered to contain antibodies against human dystrophin. The antibody titer was expressed as the highest dilution of mouse serum giving a positive response.

4.5 Results

Effects of Immunomodulation on Dystrophin Expression and Muscle Inflammation After Primary AdV-Dys Administration

Representative micrographs from different experimental groups are shown in Fig. 1. In the immunocompetent control mdx mice (Fig. 1a), there were scattered foci of inflammatory cell infiltration within those regions containing occasional dystrophin-positive fibers. Although there were greater numbers of dystrophin-positive fibers present in the anti-ICAM-1/LFA-1 (Fig. 1b), anti-CD2 (Fig. 1c) and CTLA4Ig (Fig. 1d) groups as compared to control, a considerable degree of inflammatory cell infiltration was also noted. However, the addition of FK506 to CTLA4Ig led to markedly reduced inflammatory cell invasion of dystrophin-positive regions within AdV-Dys-injected mdx muscles (see Figs. 1e & 1f). In keeping with this finding, mdx mice treated with FK506+CTLA4Ig also demonstrated substantially higher numbers of dystrophin-positive fibers, and in some instances ~90% of myofibers expressed dystrophin 30 days after primary AdV-Dys administration as shown in Fig. 1f.

The above observations were expanded upon in each group of animals through quantitative assessment of the magnitude of inflammation at 30 days post-AdV-Dys injection. These data are illustrated in Fig. 2. Surprisingly, the level of cellular inflammation in the anti-ICAM-1/LFA-1 and anti-CD2 groups was actually equal or greater than observed in immunocompetent controls, possibly due to more intense antigenic stimulation by the greater numbers of dystrophin-positive myofibers found at this time point. In contrast, the use of either CTLA4Ig or FK506 alone reduced the level of inflammation as compared to control mdx mice. Importantly, the greatest decrement in inflammatory cell invasion of myofibers after AdV-Dys injection of mdx muscles occurred in the FK506+CTLA4Ig group, where an 8-fold reduction compared to immunocompetent controls was observed. Figure 3 shows that FK506+CTLA4Ig also produced a substantially higher number of dystrophin-positive fibers at 30 days than the use of either agent in isolation. By contrast, the mean number of dystrophin-

positive fibers at 30 days after primary AdV-Dys administration to immunocompetent control mdx animals was relatively low (35 ± 5 myofibers/muscle) as previously reported (2) (29), and this was only mildly improved upon in the anti-ICAM-1/LFA-1 and anti-CD2 groups (data not shown).

Effects of Immunomodulation on Humoral Immunity to Adenovirus Capsid Proteins

Adoptive transfer of antisera obtained from both AdV-Dys-immunized and AdV-LacZ-immunized animals leads to equivalent large reductions in the efficiency of subsequent AdV-mediated dystrophin gene transfer to mdx mice (unpublished data). This suggests that antibodies generated against adenovirus capsid proteins, as well as perhaps other undefined serum factors induced by AdV administration, play a major role in reducing transduction efficiency after vector readministration to mdx muscle tissue. Accordingly, we assessed the effects of immunomodulation on production of anti-adenovirus antibodies after AdV-Dys delivery to adult mdx mouse muscles.

In sera of immunocompetent mdx mice (dilution 1:1000) examined 30 days after AdV-Dys administration, the signal for anti-adenovirus antibodies detected by ELISA amounted to ~300% of background (i.e., naive sera) values. Although the different immunomodulatory Ig tested were only able to produce a minimal blunting of this response when used in isolation, CTLA4Ig appeared to be the most effective in this regard. However, Fig. 4 shows that in contrast to the mild decrease in anti-adenovirus antibodies observed with CTLA4Ig alone, the humoral immune response against adenovirus capsid proteins was substantially reduced in the two groups of FK506-treated mice. Additionally, as was the case for cellular immunity, the reduction in humoral immune responses by FK506 was further enhanced by the addition of CTLA4Ig. In fact, the signal for anti-adenovirus antibodies obtained by ELISA in the sera of FK506+CTLA4Ig animals did not exceed background levels found in naive mdx animals not exposed to AdV-Dys.

Effects of Immunomodulation on Humoral Immunity to the Therapeutic Transgene Product

Antibodies against the transgene-encoded protein (human minidystrophin) were detected by employing an immunohistochemical assay in which serially diluted sera from AdV-Dys-injected mdx mice were reacted with sections of normal human skeletal muscle, as shown in Fig. 5. All experimental groups demonstrated the presence of anti-dystrophin antibodies at 30 days after AdV-Dys administration. In this regard, sera obtained from the immunocompetent control, anti-ICAM-1/LFA-1 and anti-CD2 groups showed detectable anti-dystrophin antibodies at dilutions exceeding 1:70000. The humoral immune response against dystrophin was less pronounced in mdx mice treated with either CTLA4Ig or FK506 alone, in which anti-dystrophin antibodies were only detectable up to dilutions of 1:35000 and 1:2500, respectively. However, in keeping with the marked reduction of anti-adenovirus antibodies described earlier, the combination of FK506+CTLA4Ig also resulted in the greatest decrease in anti-dystrophin antibodies, which were only detectable with sera diluted up to 1:200.

Effects of Immunomodulation on the Efficiency of Dystrophin Gene Transfer After Secondary AdV-Dys Administration

Given the above effects of FK506+CTLA4Ig on humoral immunity after AdV-Dys administration, we further assessed the ability of the different immunomodulatory regimens to facilitate secondary AdV-mediated dystrophin gene transfer. The mean number of dystrophin-positive fibers in the two FK506-treated groups at 10 days after AdV-Dys readministration was substantially higher than that observed in mdx mice treated with CTLA4Ig (see Fig. 6) or the other immunomodulatory Ig in isolation. Importantly, as was the case for primary AdV-Dys delivery, the addition of CTLA4Ig to FK506 led to a further major increase in the level of dystrophin expression (as compared to either CTLA4Ig or FK506 alone) after secondary AdV-Dys administration. Thus, the findings are consistent with the fact that FK506+CTLA4Ig was most effective

in achieving a global reduction in antibody generation against both adenovirus capsid proteins and dystrophin.

Figure 1. Representative micrographs of adult mdx muscles 30 days after primary AdV-mediated dystrophin gene transfer. Dystrophin immunohistochemistry was followed by hematoxylin and eosin counterstaining in the following groups: (a) Control, (b) anti-ICAM/LFA-1, (c) anti-CD2, (d) CTLA4Ig, and (e) FK506+CTLA4Ig (magnification 200x). Dystrophin-expressing myofibers are identified by dark staining of the sarcolemma, as illustrated by the straight arrows in panel (a). Examples of mononuclear inflammatory cell infiltration of dystrophin-expressing fibers are shown by the curved arrows. Note that substantial muscle inflammation was observed in all groups receiving immunomodulatory Ig alone, whereas inflammatory cell infiltration with the combination of FK506+CTLA4Ig was minimal or absent. In addition, the vast majority of muscle fibers expressed recombinant dystrophin in the FK506+CTLA4Ig group, as shown by the low-magnification (40x) micrograph in panel (f).

Figure 2. Effects of different immunomodulatory regimens on muscle inflammation after primary AdV-mediated dystrophin gene transfer. In comparison to immunocompetent control mdx animals, the level of inflammatory cell infiltration in anti-ICAM-1/LFA-1 and anti-CD2 groups was equivalent or even increased. In contrast, the use of either CTLA4Ig or FK506 alone reduced the level of inflammation. However, the greatest impact on the cellular immune response to AdV-Dys administration was observed in the FK506+CTLA4Ig group, where the degree of inflammatory cell infiltration of AdV-Dys-injected muscles was markedly abrogated as compared to immunocompetent control mice.

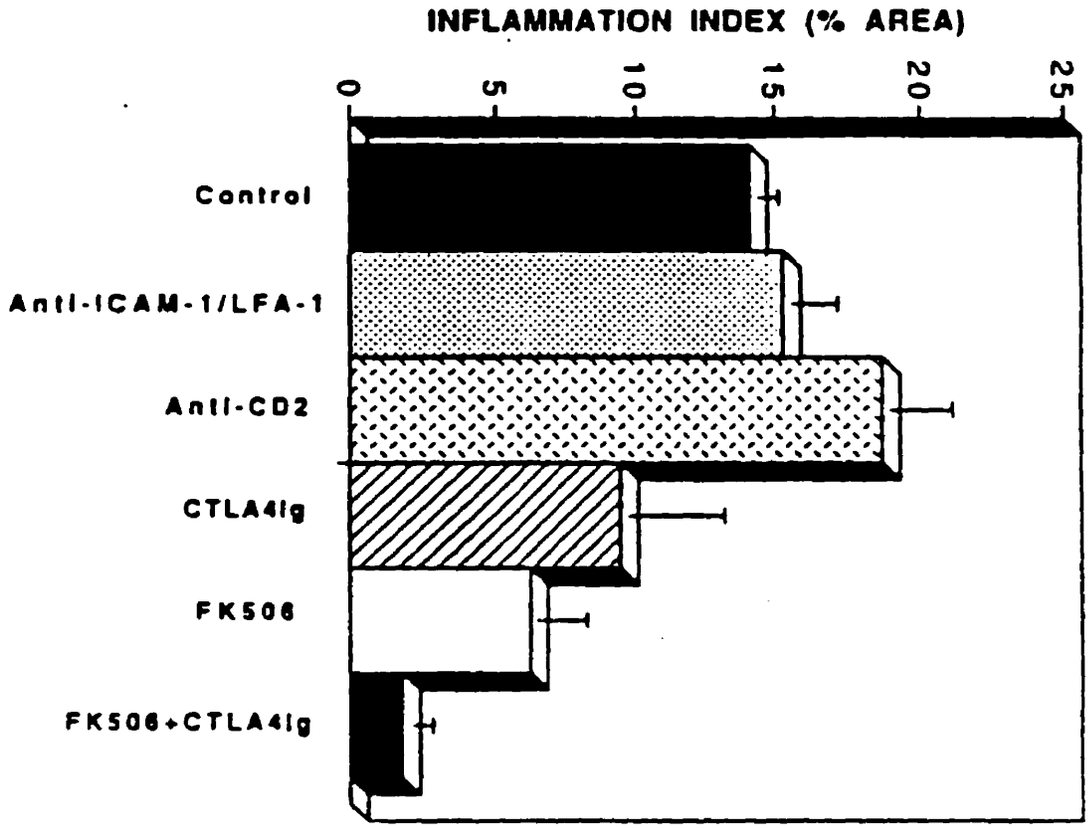

INFLAMMATION INDEX (% AREA)
0
5
10
15
20
25
Control
Anti-ICAM-1/LFA-1
Anti-CD2
CTLA4Ig
FK506
FK506+CTLA4Ig

Figure 3. Effects of immunomodulation on the level of dystrophin expression after primary AdV-Dys administration to adult mdx muscles. The total number of dystrophin-expressing myofibers on an entire cross-section of the anterior tibialis muscle was determined 30 days after AdV-Dys administration. Values are expressed as means " SE (n=3-4 animals/group). As can be seen, the combination of FK506+CTLA4Ig led to a major increase in transduction efficiency over that attained when either CTLA4Ig or FK506 was used in isolation.

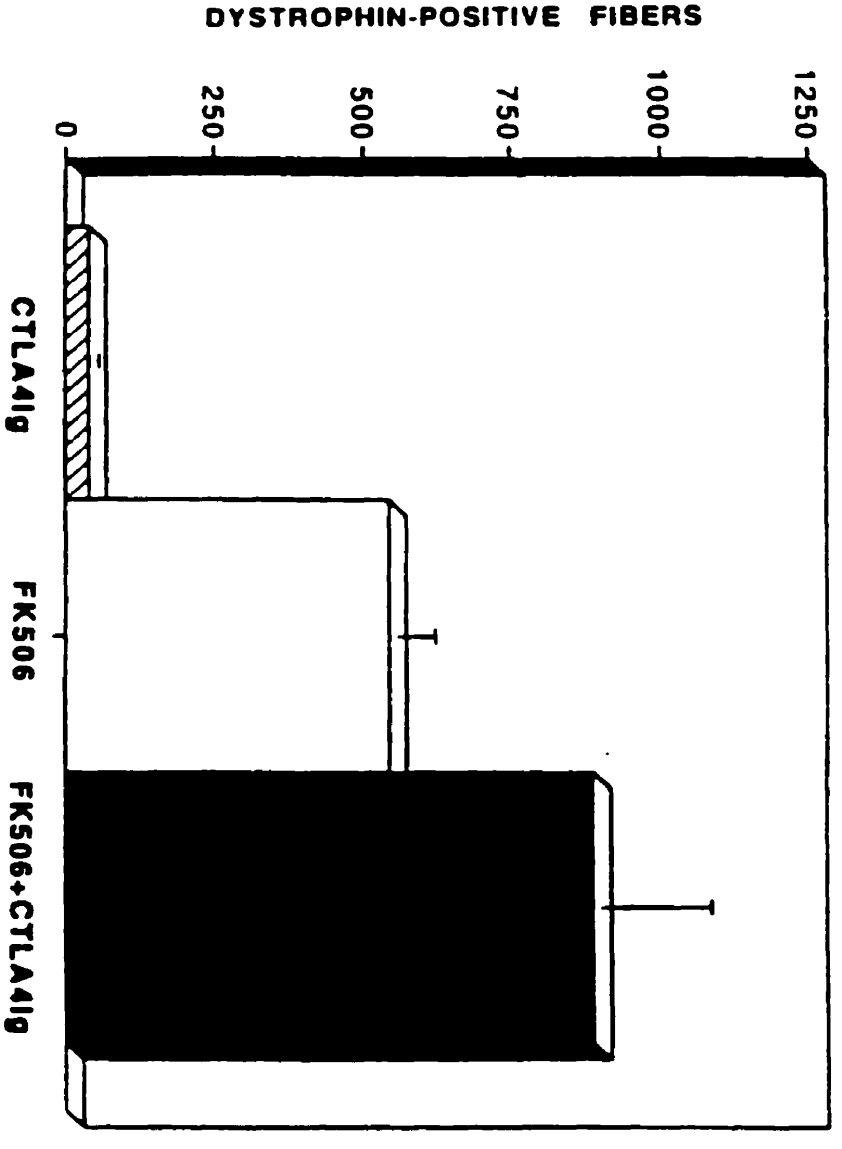

DYSTROPHIN-POSITIVE FIBERS
0
250
500
750
1000
1250
CTLA4Ig
FK506
FK506+CTLA4Ig

Figure 4. Effects of immunomodulation on anti-adenovirus antibody production after AdV-mediated dystrophin gene transfer to adult mdx muscles. Anti-adenoviral antibodies were quantitated by ELISA and are expressed as a percentage of background values obtained from a negative control (i.e., naive non-AdV-injected) mdx mouse. All data are mean values ± SE (n=3-4 animals/group). Primary AdV-Dys administration (day 0) was followed by secondary administration on day 20, and sera from mdx mice were then obtained on day 30. The combination of FK506+CTLA4Ig achieved the greatest reduction of the humoral immune response against adenoviral capsid proteins. Open bars, 1:1,000 dilution; closed bars, 1:10,000 dilution.

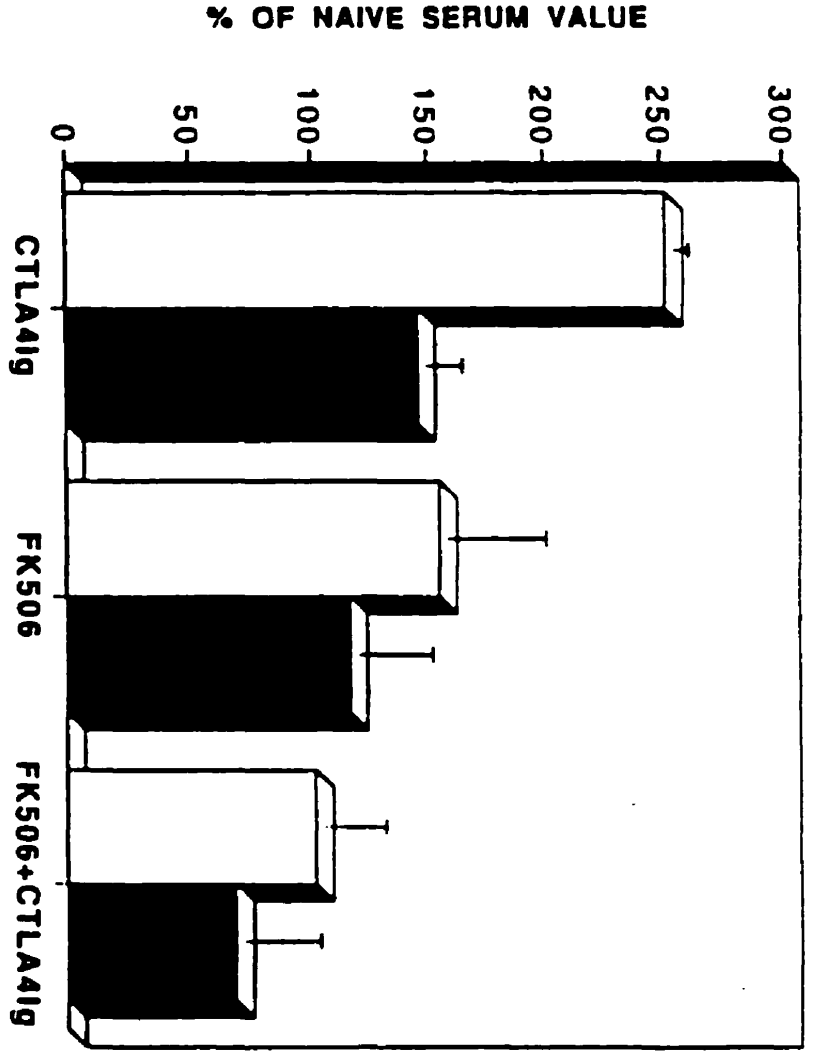
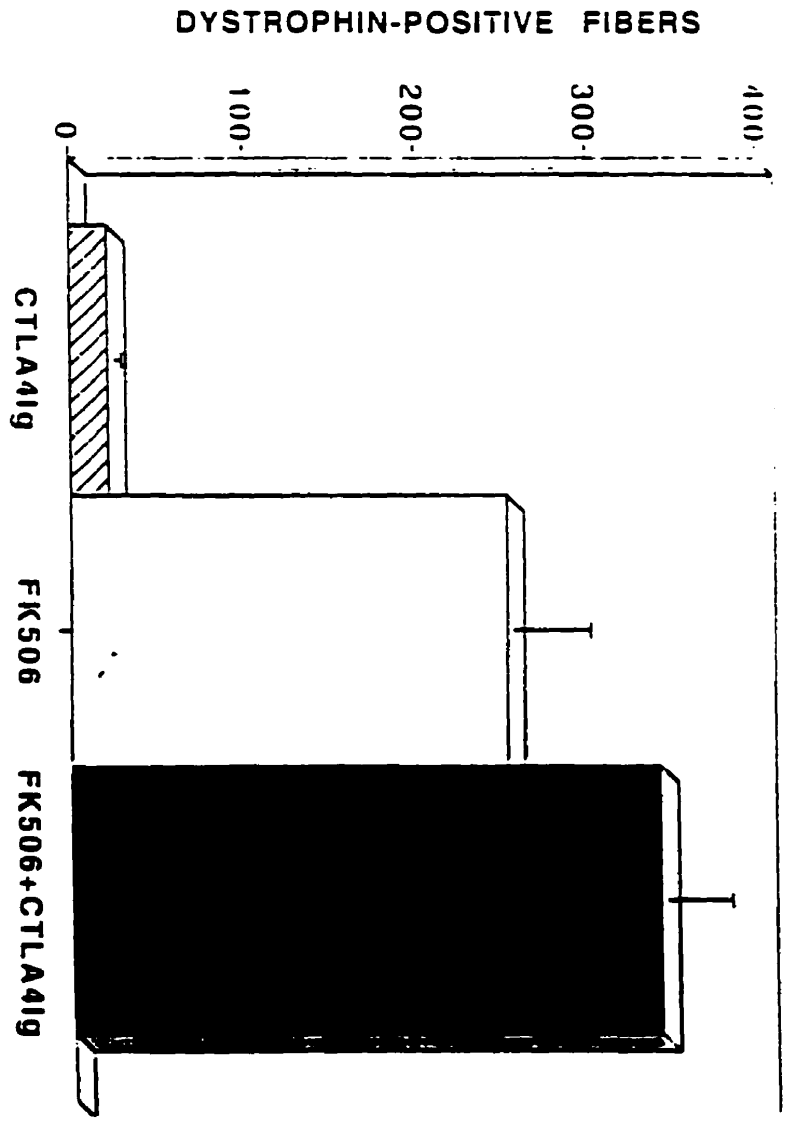

% OF NAIVE SERUM VALUE
0
50
100
150
200
250
300
CTLA4Ig
FK506
FK506+CTLA4Ig

Figure 5. Representative micrographs illustrating immunohistochemical detection of antibodies generated against human dystrophin following AdV-Dys delivery to adult mdx mice. Pooled sera from the different experimental groups were serially diluted and reacted with sections of normal human skeletal muscle (magnification 400x): (a) Naive mdx (i.e., non-AdV-injected) mouse sera (1:400 dilution) generated no sarcolemmal staining, consistent with an absence of antidystrophin antibodies; (b) Control mdx (AdV-Dys-injected without immunosuppression) sera, on the other hand, produced strong sarcolemmal staining at a dilution of 1:35000, consistent with a high level of antidystrophin antibodies; (c) CTLA4Ig-treated mdx sera allowed only very faint sarcolemmal staining at the same 1:35000 dilution; (d) FK506+CTLA4Ig-treated mdx sera generated no detectable sarcolemmal staining at a dilution of 1:400, similar to the naive group shown in (a).

Figure 6. Effects of immunomodulation on dystrophin expression after secondary AdV-mediated gene transfer to adult mdx mice. The total number of dystrophin-expressing myofibers on an entire cross-section of the anterior tibialis muscle was determined 10 days after AdV-Dys readministration. Values are expressed as means " SE (n=3-4 animals/group). As was the case after primary AdV-Dys administration, the combination of FK506+CTLA4Ig achieved the highest transduction efficiency following secondary AdV-mediated dystrophin gene transfer to adult mdx muscles.


DYSTROPHIN-POSITIVE FIBERS
0
100
200
300
400
CTLA4Ig
FK506
FK506+CTLA4Ig

4.6 Discussion

This is the first study to compare the ability of different immunomodulatory Ig to facilitate effective primary as well as secondary AdV-mediated dystrophin gene transfer to dystrophic mouse muscle tissue. The short-term administration of neutralizing Ig directed against cell adhesion molecules during the period corresponding to initial AdV capsid particle exposure and early dystrophin transgene expression could theoretically prevent molecular interactions normally required for CD3-T cell receptor-mediated recognition of these foreign antigens. Administration of CTLA4Ig, on the other hand, should not interfere with antigen recognition, but rather with the subsequent step of costimulation that is generally needed to achieve optimal T cell activation and clonal expansion. Both of these strategies by themselves have previously been demonstrated to successfully induce specific Atolerance@ to allografted organs in experimental animals (19) (4;25;26), thus raising the possibility that a state of tolerization to both vector proteins and dystrophin might also be achievable.

In the present study, short-term interference with cell adhesion and costimulatory molecules was only able to partially abrogate undesirable immune responses to AdV-mediated dystrophin gene transfer. Indeed, the equal or even greater degree of muscle inflammation observed in the anti-ICAM-1/LFA-1 and anti-CD2 groups as compared to control mdx animals at one month after AdV delivery suggests that these treatments simply delayed the onset of cellular immunity against AdV-infected myofibers. However, among the immunomodulatory Ig regimens examined, CTLA4Ig was found to be the most effective in blunting cellular and humoral immune responses resulting from AdV-mediated gene transfer. In addition to the present study, two other groups have reported on the effects of CTLA4Ig administration in the context of AdV-mediated gene transfer (23) (14) (24). Guerette et al. (14) reported a low efficacy of CTLA4Ig in preventing cellular and humoral immune responses after AdV-mediated transfer of the LacZ reporter gene to non-dystrophic murine skeletal muscles. Kay et al. (23) (24), on the other hand, found that CTLA4Ig prevented cellular infiltration and

allowed prolonged transgene expression for several months after liver-directed AdV-mediated transfer of the human a-1 antitrypsin gene. Reported differences among studies in the immunosuppressive effects of CTLA4Ig after AdV-mediated gene transfer may be related to a number of factors. First, different intrinsic immunogenicities of the transgene products examined likely played an important role in determining the intensity of ensuing immune responses. Second, the use of murine CTLA4Ig (23;24) may offer greater therapeutic advantage in mouse models. In particular, at the latest time point (day 10) in which human CTLA4Ig was administered in the present study, generation of anti-human neutralizing antibodies could theoretically have been sufficient to limit its effectiveness. Therefore, it is possible that murine CTLA4Ig would have been even more efficacious in preventing AdV-triggered immune responses when used alone as well as in combination with FK506. Along these same lines, a lack of neutralizing antibodies against the murine analogue could also permit more prolonged treatment with CTLA4Ig, although it should be noted that this strategy did not appear to offer any additional benefit in the context of liver-directed AdV-mediated gene transfer (24).

The nature of the host immune response to AdV delivery can also vary as a function of the AdV-injected target tissue being studied (21;48). This may be related to different modes of antigen presentation and priming of T cell subsets in the different organ systems, and could even differ between healthy and dystrophic skeletal muscles since the latter contain numerous macrophages that could act as professional APCs. Under these conditions, it is conceivable that AdV infection of resident macrophages within dystrophic muscle could amplify cellular as well as humoral immune responses. Whereas cellular immunity directed against adenoviral proteins alone appears able to destroy AdV-infected cells in lung (49) and liver (45), in skeletal muscle it has been suggested that adenoviral antigens are of little importance in this regard (40;44). This conclusion was based upon the observation that animals showing natural immunological tolerance to transgene-encoded proteins did not demonstrate destructive cellular immune responses against AdV-infected myofibers (40;44).

Given this apparent predominance of transgene-encoded proteins as targets of the CTL attack after AdV infection of skeletal muscle (40;44), a noteworthy finding in this study was the highly immunogenic nature of the human dystrophin protein when expressed in adult mdx mice. Since dystrophin normally maintains an intracellular location, in the present study it is likely that necrosis of AdV-infected cells (either due to CTL attack or incomplete protection from the underlying disease process as a result of subtherapeutic recombinant dystrophin levels) allowed for dystrophin exposure to the extracellular milieu with subsequent antibody formation. This occurred despite the presence in mdx muscles of a small number (<1%) of Arevertant@ fibers able to express murine dystrophin due to somatic cell backmutations (presumably during embryonic development) of the gene (18), which could theoretically confer some degree of immunological tolerance to exogenously supplied dystrophin. Although the lack of tolerance to human dystrophin observed in our study could be related to species differences in dystrophin protein structure, it should be noted that anti-dystrophin antibodies have also been documented after murine dystrophin gene transfer to mdx mice via myoblast transplantation (41). The present study cannot resolve the question of whether anti-dystrophin antibodies played a direct role in the eventual elimination of AdV-infected fibers by way of antibody-dependent cellular cytotoxicity (ADCC) (3). However, it has been reported that the presence of anti-dystrophin antibodies per se does not appear to produce an accelerated loss of dystrophin-positive fibers after transplantation of normal murine myoblasts to mdx mice (41).

The immunosuppressive compounds cyclosporine and FK506 act by binding to members of the immunophilin class of proteins (28). The resulting drug-immunophilin complexes interfere with T cell signaling events via calcineurin required for lymphocyte activation after stimulation of the CD3-T cell surface receptor (11;22;28). There is now extensive clinical experience with these agents, which have been used primarily as a means of preventing the rejection of transplanted organs. Unfortunately, in order to achieve adequate levels of immunosuppression it is frequently necessary to employ drug doses

that also cause a degree of organ toxicity. To minimize such problems in the context of AdV-mediated gene transfer, it would be highly desirable to develop alternative strategies that could be used to enhance the level of immunosuppression without incurring an increase in adverse effects. The use of CTLA4Ig is particularly attractive in this regard, as it involves no apparent toxicity and has the additional advantage of allowing potential synergistic immunomodulatory effects, since its mechanism of action is distinct from the CD3-T cell receptor-triggered pathway targeted by immunophilin-binding drugs (22) (11). In support of this concept, the addition of CTLA4Ig to anti-CD40 ligand antibody treatment has recently been reported to enhance the efficiency of primary as well as secondary AdV-mediated gene transfer to mouse liver, whereas blockage of either the B7:CD28 or CD40:CD40 ligand pathways by themselves was considerably less effective (24).

In the present study, we demonstrate that despite the use of essentially maximal FK506 therapy (approximately 10x the usual clinical dose), superimposed inhibition of the B7:CD28 costimulatory pathway with CTLA4Ig produced a further major blunting of cellular as well as humoral immune responses directed against both adenovirus capsid proteins and recombinant dystrophin. Furthermore, the benefits of utilizing a combinatorial strategy to block both calcineurin and CD28 signaling pathways were observed after primary as well as secondary AdV-mediated dystrophin gene transfer. It should be noted that although the combination of FK506 and CTLA4Ig was able to reduce anti-adenovirus antibodies to essentially undetectable levels with an accompanying improvement in secondary gene transfer, the level of myofiber transduction after secondary AdV-Dys administration was nonetheless lower than that attained following the initial AdV-Dys injection. This is consistent with previously reported findings in CD40 ligand-deficient mice (48), which also demonstrated diminished secondary transduction in liver despite a failure to develop neutralizing anti-adenoviral antibodies. Therefore, it is possible that in addition to neutralizing antibodies, other serum factors (e.g., cytokines (50)) also play a role in reducing

secondary transduction efficiency and could thus serve as further targets for future therapeutic modulation of specific immune system components.

While it might be argued that vector modification is preferable to host immunosuppression as a means of preventing or mitigating undesirable immunological responses to AdV-mediated gene transfer, it is important to recognize certain inherent limitations to the former approach. As discussed earlier, there is accumulating evidence that in many instances the transgene product rather than adenoviral gene products represent the primary target of the CTL response that leads to the eventual loss of therapeutic gene expression (40;44). Therefore, given our results indicating dystrophin itself to be highly immunogenic in the context of AdV-mediated gene transfer to dystrophin-deficient animals, one would predict that strategies involving either inactivation (47) or deletion (5;12;16) of adenoviral genes from the vector backbone are unlikely to be completely effective in allowing long-term persistence of dystrophin expression. Indeed, early experience with adenoviral vectors that are lacking in all viral genes appears to confirm the above concern (5;16). A particularly interesting development is the recent report that recombinant adeno-associated virus (AAV) vectors efficiently transduce mature skeletal muscle fibers without eliciting an immune response against transgene products that are, by contrast, immunogenic in the context of AdV-mediated gene transfer (10;43). This suggests that AdV particles may actually act as an adjuvant and thereby boost the immune response against transgene products, including dystrophin. Application of AAV vectors to the treatment of DMD is limited, however, by a relatively small insert capacity of about 5 kb (43). Although it may be possible to further reduce the size of the current dystrophin minigene and its associated promoter elements so that these can be accomodated by the AAV vector, it is unknown whether the resulting severely truncated dystrophin protein would be functional. In addition, for both AdV (23;46) and AAV (10;43), the problem of humoral immunity against input viral capsid proteins and consequent inhibition of secondary transduction remains problematic in the absence of immunosuppressive therapy.

In summary, we have tested a number of strategies for providing effective immunosuppression after AdV-mediated dystrophin gene transfer in adult dystrophic (mdx) mice. Whereas interference with adhesion cell molecule function or B7:CD28 costimulation in isolation is only mildly effective in blocking undesirable immune responses, combined inhibition of CD3-T cell receptor-mediated signaling via calcineurin and B7:CD28 costimulation markedly diminishes host immunity against both vector proteins and dystrophin. Based on the results obtained in this study, we speculate that such an approach might permit repetitive AdV-Dys administration to previously targeted muscles, thereby potentially allowing a stepwise augmentation of the level of dystrophin gene expression in dystrophic muscle tissues. Additional studies are currently in progress to test this hypothesis as well as to assess whether this strategy will lead to commensurate improvements in muscle contractile function.

4. 7 ACKNOWLEDGMENTS

This investigation was supported by grants from the Medical Research Council of Canada, the Muscular Dystrophy Association of Canada, the Muscular Dystrophy Association, Inc. (USA), the National Research Council of Canada, and the Association Pulmonaire du Quebec. G.-H. Guibinga is a recipient of a studentship award from the Montreal Chest Institute. H. Lochmuller was supported by grants from the Deutsche Forschungsgemeinschaft and Sander-Stiftung, Germany. J. Nalbantoglu is a Research Scholar of the Fonds de la Recherche en Sante du Quebec. B.J. Petrof is the recipient of a Clinician-Scientist Award from the Medical Research Council of Canada. We are grateful to Dr. P. Linsley for the gift of CTLA4Ig, and Dr. P. Altevogt for the gift of hybridoma 12-15. We thank N. Chughtai, N. Matusiewicz, S. Prescott and C. Allen for expert technical assistance.

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CHAPTER 5

Overexpression of dystrophin and utrophin by adenovirus-mediated gene transfer produces differential effects on muscle function in adult immunocompetentent dystrophic (mdx) mice

5.1 Prologue

In the precedent chapter we have demonstrated that a combined immunosuppressive strategy permits to facilitate the primary and secondary AdV-mediated dystrophin gene transfer in dystrophic muscles. Although immunosuppressive regimens are now a routine procedure in clinical medicine, they are tremendously expensive and are associated with complications. Therefore in the present chapter, we have assessed the ability of utrophin (or formerly known as dystrophin related protein) to act as a surrogate of dystrophin, in gene replacement therapy for dystrophin deficient muscles. Utrophin and dystrophin have high degree of homology and can accomplish overlapping function within the muscle cells. This includes the binding of actin and dystrophin protein complex. In addition, it has been reported that in normal fetal muscle, utrophin is localized to the sarcolemma before being replaced gradually by increasing levels of dystrophin during the development. Importantly utrophin is expressed in DMD as well as dystrophic (mdx) mice is several organs, it does not therefore constitute a neoantigen in the setting of dystrophin deficiency. Consequently in the current chapter we are comparing the efficacy of utrophin versus dystrophin transgene delivery on dystrophic muscle function at different stages of host maturity. We are reporting that in context of little pre-existing muscle damage and reduced immune response as evidenced in neonatal animals AdV-mediated utrophin and AdV-mediated dystrophin gene transfer are therapeutically equivalent. By contrast in immunocompetent adult dystrophic mice, utrophin minigene provided through AdV has an obvious superior therapeutic potential comparatively to AdV-minigene. Based upon the reported findings, utrophin gene transfer could be envisaged as an alternative therapeutic avenue for DMD.

5.2 Abstract

Duchenne muscular dystrophy (DMD) is a fatal disease caused by defects in the gene encoding dystrophin. Dystrophin is a cytoskeletal protein, which together with its associated protein complex helps to protect the sarcolemma from mechanical stresses associated with muscle contraction. Gene therapy efforts aimed at supplying a normal dystrophin gene to DMD muscles could be hampered by host immune system recognition of dystrophin as a foreign protein. In contrast, a closely related protein called utrophin is not foreign to DMD patients, and is able to compensate for dystrophin deficiency when overexpressed throughout development in transgenic mice. However, the issue of which of the two candidate molecules is superior for DMD therapy has remained an open question. In this study, dystrophin and utrophin gene transfer effects on dystrophic muscle function were directly compared in the murine (mdx) model of DMD using E1/E3-deleted adenovirus vectors containing either a dystrophin (AdV-Dys) or a utrophin (AdV-Utr) transgene. In immunologically immature neonatal animals, AdV-Dys and AdV-Utr improved tibialis anterior muscle histopathology, force-generating capacity, and the ability to resist injury caused by high-stress contractions to an equivalent degree. By contrast, only AdV-Utr was able to achieve significant improvement in force generation and the ability to resist stress-induced injury in the soleus muscle of immunocompetent mature mdx animals. In addition, in mature mdx mice there was significantly greater transgene persistence and reduced inflammation with utrophin as compared to dystrophin gene transfer. We conclude that dystrophin and utrophin are largely equivalent in their intrinsic abilities to prevent the development of muscle necrosis and weakness when expressed in neonatal mdx animals with an immature immune system. However, because immunity against dystrophin places an important limitation on the efficacy of dystrophin gene replacement in an immunocompetent mature host, the use of utrophin as an alternative to dystrophin gene transfer in this setting appears to offer a significant therapeutic advantage.

5.3 Introduction

Duchenne muscular dystrophy (DMD) is the most common X-linked fatal disorder in man. The primary defect is the absence of dystrophin, a subsarcolemmal structural protein which plays an important role in maintaining the physical integrity of the muscle cell surface membrane (16;43). Dystrophin is part of a complex of proteins that links the internal cytoskeleton of muscle fibers to the extracellular matrix (19;43). Disruption of this linkage renders muscle fibers abnormally susceptible to contraction-induced plasma membrane damage (31). Viral vectors, and those based on replication-defective recombinant adenovirus (AdV) in particular, are considered a promising method for delivering a functional dystrophin gene to dystrophic muscle. However, a major limitation to the implementation of this strategy using first-generation vectors is that AdV-mediated gene expression in skeletal muscles of mature animals is rapidly abolished by host cellular immunity. This immune response involves elimination of AdV-infected myofibers by CD8+ cytotoxic T lymphocytes (46), and leads not only to a loss of therapeutic gene expression, but also to an attendant worsening of muscle contractile function (29;30).

It was initially assumed that the observed stimulation of host T cells following AdV administration was triggered mainly by host recognition of vector-derived viral antigens expressed at low levels in infected cells (46). However, there is now considerable evidence that cell-mediated immunity can be directed primarily against therapeutic transgene products rather than viral proteins (26;37;45), particularly in the case of genetic null mutations in which the missing therapeutic protein is by definition foreign to the host. Therefore, for DMD patients in whom there is a lack of dystrophin, there is concern that forced expression of a non-self epitopes associated with the therapeutic dystrophin protein could not only compromise long-term dystrophin gene expression but also worsen muscle function by stimulating cytotoxic T cell-mediated destruction of the dystrophin-expressing myofibers. Although this

problem could in principle be dealt with through the imposition of broad-based host immunosuppression, a far more attractive option would be to employ an alternative, non-immunogenic therapeutic transgene.

Utrophin, the autosomally-encoded homologue of dystrophin (formerly called dystrophin-related protein, DRP), is a promising candidate for such an approach. Like dystrophin, utrophin is capable of providing structural linkage between the actin-based cytoskeleton and the extracellular matrix via binding to the dystrophin-associated protein complex (DPC) spanning the membrane of muscle fibers (36). Four structural domains are common to both utrophin and dystrophin: 1) the N-terminus containing attachment sites for cytoskeletal F-actin; 2) the rod domain containing spectrin-like repeats; 3) the cysteine-rich domain containing the binding site for β-dystroglycan, which is critical for ultimate linkage to laminin-2 in the extracellular matrix; and 4) the C-terminus containing binding sites for syntrophin and dystrobrevin. Importantly, dystrophin-deficient X-linked muscular dystrophy (mdx) mice overexpressing a truncated utrophin transgene in muscle have been reported to show a reduction of dystrophic histopathology as well as improved muscle performance (7;36). In addition, mice deficient in both utrophin and dystrophin demonstrate a more severe dystrophic phenotype than mice lacking in either protein alone (6;10). Utrophin expression is found in a large number of tissues and cell types in the body (21;23) as well as in the muscles of DMD patients (15;20), although the latter is obviously at insufficient levels to maintain the integrity of muscle fibers.

Given that utrophin does not constitute a neoantigen in dystrophin-deficient muscles, it has been proposed that utrophin overexpression in dystrophic myofibers (either through upregulation of the endogenous utrophin gene or vector-mediated utrophin gene transfer) could permit adverse immunological responses inherently associated

with dystrophin gene replacement in a dystrophin-deficient host to be averted. However, although utrophin offers a theoretical advantage over dystrophin in this regard, the above hypothesis has never been tested. Studies performed to date in transgenic-mdx mice have been unable to address this issue because overexpression of either dystrophin or utrophin occurs in concert with immunological development in this setting, thereby leading to any transgene product being considered a Aself@ protein by the host immune system. Moreover, it should be noted that differences in the therapeutic efficacy of dystrophin and utrophin in muscles with prior necrosis could not be evaluated in such transgenic mice, since pathologic features were preempted (as opposed to treated) in these animals through forced expression of the therapeutic protein from birth. In a similar fashion, although we have previously reported that AdV-mediated utrophin gene transfer leads to a reduction in mdx muscle pathology and an increased resistance to contraction-induced muscle injury when compared to untreated mdx muscles (8;9), functional studies were limited to immunologically immature mdx animals. In addition, no direct comparison between AdV-mediated dystrophin and utrophin gene transfer has been performed in dystrophic animals of any age group.

Therefore, in order to more directly assess the relative merits of dystrophin versus utrophin gene transfer as a treatment modality for DMD, in this study we have utilized AdV-mediated gene transfer to perform the first direct, head-to-head comparison of these two candidate molecules for gene therapy of DMD under experimental conditions that were identical in all other respects. The data reported here point to complex interactions that differentially affect the ability of dystrophin or utrophin overexpression to attain functional amelioration in dystrophin-deficient muscles. A point of particular importance is that in contrast to prior studies (7;8;36) in which disease manifestations were averted by overexpressing these therapeutic genes

during embryonic development or at a very early age after birth, the present study also involved treatment of adult mdx mice with a fully competent immune system and established muscle pathology. This situation is analogous and therefore most directly relevant to the anticipated clinical scenario in DMD patients.

5.4 Material and Methods

Preparation of Recombinant Adenoviruses

Adenovirus recombinants containing either the 6.3 kb dystrophin (AdV-Dys) Becker minigene or a 6 kb utrophin (AdV-Utr) minigene expression cassettes were constructed using E1/E3-deleted replication-defective serotype 5 human adenovirus as previously outlined in detail (1;9). Both cDNAs were of human origin with the exception of the first 2 kb of utrophin, which contained murine sequence. In both vectors, the insert cDNA was driven by cytomegalovirus (CMV) promoter/enhancer elements inserted into the E1 region. The absence of E1- containing, replication-competent AdV was confirmed with a sensitive PCR screening assay (22), using primers homologous to nucleotides 1333 to 1350 and 1761 to 1745 of serotype 5 human AdV (5'- CCTGTGTCTAGAGAATGC-3', 5'-CAAGTTACGCACAGCAG-3'). Total AdV particle number titers were determined by measuring optical density in a spectrophotometer at 260 nm. Infectious titers were equivalent for AdV-Dys and AdV-Utr as determined by cytopathic effects on 293 cells.

Animals and Surgical Procedures

Dystrophin-deficient mdx mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg) by intramuscular (i.m.) injection prior to AdV injection. Selection of the muscles and time points examined as well as AdV dosage were based upon our previous studies of AdV-mediated dystrophin (1)or utrophin (8) gene transfer in the respective age groups. Immunocompetent adult mdx mice (45-60 days old) received injections of AdV-Dys or AdV-Utr (diluted in 15 µl saline, total particle number of 1.5 x 10^9) into the soleus hindlimb muscle on one side, with the contralateral saline-injected side providing a within-animal control. Adult mdx mice were euthanized at 10 and 60 days after AdV administration. Neonatal mdx mice (3-5 days old) were

used to compare the response to AdV-Dys or AdV-Utr administration in animals with an immature immune system (3) and an early stage of muscle pathology (24). Because the soleus was too small to accurately inject in neonatal animals, AdV injections were made into the larger tibialis anterior muscle (diluted in 5 µl saline, total particle number of 3.5×10^9); the contralateral muscle was again injected with saline alone to serve as a control. The neonatally-injected mice were euthanized at 30 days post-injection of AdV.

Measurement of Isometric Muscle Mechanics

Mice were first anesthetized to achieve a loss of deep pain reflexes. AdV-injected and control muscles were then carefully removed in random order to determine in vitro isometric contractile properties as previously described (44). Briefly, muscles were mounted vertically in a jacketed tissue bath chamber filled with Ringer's solution (composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$ and 6 uM d-tubocurarine chloride) continuously perfused with 95% O2:5% CO2 (pH 7.4) and maintained at 25^0C; a thermoequilibration period of 10 min was observed prior to initiating contractile measurements. One end of each muscle was securely anchored to a platform near the base of the chamber, while the opposite tendon was tied to the lever arm of a force transducer/length servomotor system (Model 300B dual mode, Cambridge Technology, Watertown, MA); in the case of the tibialis anterior muscle of neonatally-injected animals, the proximal end of the muscle remained attached to the bone. A mobile micrometer stage (Newport Instruments, Toronto, Canada) was employed to allow incremental adjustments of muscle length. Electrical field stimulation was induced via platinum plate electrodes placed into the bath on both sides of the muscle. Supramaximal stimuli with a monophasic pulse duration of 2 ms were delivered using a computer-controlled electrical stimulator (Model S44, Grass Instruments, Quincy, MA) connected in series

to a power amplifier (Model 6824A, Hewlett Packard, Palo Alto, CA). Muscle force was displayed on a storage oscilloscope (Tektronix, Beaverton, OR), and the data were simultaneously acquired to computer (Labdat/Anadat software, RHT-InfoData, Inc., Montreal, Canada) via an analog-to-digital converter at a sampling rate of 1000 Hz for later analysis. After adjusting each muscle to optimal length (Lo, the length at which maximal force is achieved), five twitch stimulations were recorded and the mean value was used to determine maximal isometric twitch force (Pt). The muscles were then sequentially stimulated at frequencies of 30, 60, 90, and 120 Hz for 1 s each, with 2 minutes between each contraction; this allowed maximum isometric tetanic force (Po) to be determined. Muscles were then removed from the bath and Lo was directly measured under a dissecting microscope with microcalipers accurate to 0.1 mm. Total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.06 g/cm^3). This allowed specific force (force/cross-sectional area) to be calculated, which was expressed as N/cm^2.

Assessment of Muscle Susceptibility to Contraction-Induced Mechanical Stress

Myofibers lacking in dystrophin are abnormally susceptible to damage triggered by mechanical stresses associated with muscle contraction (31). Therefore, after allowing a 10-min recovery period following the above measurements of isometric contractile properties, AdV-injected and control muscles were compared to determine their ability to withstand high-stress lengthening (eccentric) contractions. A variation of the protocol has been described in detail elsewhere (31). Briefly, each muscle was supramaximally stimulated at 90 Hz for 1700 ms; the muscle was held at Lo (isometric) during initial 1000 ms, lengthened through a distance of 5 % (for neonatally-injected muscles) or 15% (for adult-injected muscles) Lo during the subsequent 250 ms, and maintained at the final length during the last 450 ms of

stimulation. A total of five such stimulations were performed, each being separated by a 2-min recovery period at Lo. The mean peak force (P_{PEAK}) and force-time integral attained during the eccentric portion of the contraction ($\int P_{ECC}.dt$) were measured and used as indices of mechanical stress imposed upon the muscles (40). The percent declines in maximal isometric force (Po) and maximum rate of force development (dP/dt) from the first to the last contraction (measured from the isometric component at Lo) served as functional indicators of contraction-induced mechanical injury to muscle as previously described (31;40).

Dystrophin/Utrophin Immunostaining and Histopathology

After completing muscle mechanics studies, the muscles were embedded in mounting medium and snap-frozen in isopentane pre-cooled with liquid N_2. Transverse sections (6 μm thick) were obtained in a cryostat and then fixed on slides in 1% acetone. Immunohistochemical procedures were carried out to detect dystrophin expression using a polyclonal antidystrophin (C-terminus) primary antibody as previously described (1). Utrophin immunostaining was accomplished by blocking with affinity-purified goat anti-mouse IgG Fab fragment (Jackson ImmunoResearch, West Grove, PA) and incubation with a primary monoclonal antibody directed against the N-terminus of utrophin (Novocastra Laboratories, Newcastle Upon Tyne, UK) followed by reaction with a Cy3-conjugated affinity-purified goat anti-mouse IgG (9). Muscle sections were also counterstained with hematoxylin/eosin (H/E) to allow determination of the prevalence of centrally nucleated myofibers, an indicator of the degree of prior necrosis and regeneration (1;8). Microscopically visualized sections were photographed using a video camera and the image was stored on a Macintosh computer. Analysis of the number of dystrophin- or utrophin-positive myofibers (i.e., only those myofibers demonstrating complete circumferential sarcolemmal staining, with or without associated cytoplasmic reactivity) on the entire muscle cross-section

was performed using the public domain program NIH Image (http://rsb.info.nih.gov/nih-image). The above analysis was done independently by two different observers blinded to the identity of the samples and the results obtained were then averaged.

In order to quantify the magnitude of inflammation in AdV-injected as well as control muscles, a standard point-counting technique was employed and the area fraction of inflammation was determined as previously described (11). Briefly, 3-4 randomly selected microscopic fields per muscle were selected, and a 100-point grid was superimposed onto each captured image using a stereology software package (Stereology Toolbox, Morphometrix, Davis, CA). An abnormal point was defined as either falling upon inflammatory cells or a myofiber invaded by such cells. The area fraction of inflammation was calculated by dividing the number of abnormal points by the total number of points falling on the tissue section, and is expressed as a percentage.

Statistical Analysis

Differences between AdV-injected and contralateral control saline-injected muscles were determined using Student's two-tailed *t* test for dependent samples, whereas differences between AdV-Dys and AdV-Utr groups were assessed using Student's two-tailed *t* test for independent samples. In the case of multiple comparisons, the Bonferonni correction was applied. All values are means ± SE. Statistical significance was defined as $p<0.05$.

5.5 Results

I. Comparative Effects of AdV-Dys and AdV-Utr in Immunologically Immature (Neonatally-Injected) Dystrophic Mice

Maintenance of transgene expression

Figure 1 shows representative photomicrographs of mdx muscles 30 days after AdV-mediated gene transfer during the neonatal period, while the corresponding group mean quantitation for total numbers of dystrophin- and utrophin-expressing myofibers is depicted in Fig. 2. Myofibers expressing dystrophin averaged 375 $\pm$ 81 (30 $\pm$ 4% of total myofiber number) within the AdV-Dys-injected muscles. Control muscles not receiving AdV-Dys obtained from the same animals, on the other hand, only showed very rare dystrophin-positive myofibers (averaging 0.2 $\pm$ 0.1% of total fiber number); these are so-called "revertant" fibers previously noted in mdx mice, which are presumed to represent somatic cell backmutations of the endogenous dystrophin gene (17). In AdV-Utr-injected muscles, utrophin-overexpressing myofibers (identified by circumferential sarcolemmal or cytoplasmic staining rather than the normally restricted pattern of utrophin expression at the neuromuscular junction) amounted to 374 $\pm$ 41 (34 $\pm$ 4% of total myofibers within the muscle); this value did not differ significantly from that obtained for dystrophin-expressing fibers after AdV-Dys administration. Because endogenous utrophin expression is upregulated to a certain degree in mdx muscles (15;20), control muscles not receiving AdV-Utr also exhibited a background level of utrophin-overexpressing fibers, but the proportion of such fibers was markedly lower (8.0 $\pm$ 1.5% of total myofibers) than observed in the AdV-Utr-injected muscles.

Dystrophic histopathology

Table 1 shows the percentage of fibers with central nuclei in AdV-Dys- and AdV-Utr-injected tibialis anterior muscles at 30 days following vector delivery to neonatal mdx mice. Centrally located nuclei are characteristic of myofibers that have regenerated

after an episode of necrosis, and quantitation of the proportion of fibers with central nucleation can therefore provide an index of the level of antecedent muscle necrosis (1;8). In both AdV-Dys- and AdV-Utr-injected muscles, there was a highly significant reduction in the percentage of centrally nucleated myofibers in comparison to the contralateral untreated control muscles, consistent with a protective effect against muscle necrosis derived from AdV-mediated dystrophin or utrophin gene transfer. There was no significant difference in central nucleation between muscles treated with AdV-Dys and AdV-Utr.

Isometric muscle mechanics

Isometric force parameters in AdV-Dys- and AdV- Utr-injected muscles are provided in Fig. 3, and are expressed as a percentage of the contralateral control muscle values. As can be seen, maximal tetanic force generation was significantly greater in AdV-Dys-injected muscles than in untreated control muscles. A strong trend toward greater tetanic force production was also found in AdV-Utr-injected muscles as compared to controls, although this did not achieve statistical significance ($p=0.15$). There was no significant difference in force-generating capacity between AdV-Dys- and AdV-Utr-injected muscles.

Susceptibility to high-stress contractions

Because such stress-induced injury to muscle is manifested by reductions in both maximal tetanic force (Po) and the rate of force development (dP/dt) (31), the effects of AdV-Dys and AdV-Utr administration on these parameters were determined and normalized to the magnitude of mechanical stress (as reflected by P_{PEAK} and $\int P_{ECC}.dt$) placed on the muscle (see Fig. 4). As shown in Fig. 5, there was a significantly lower decline in Po in muscles treated with AdV-Dys or AdV-Utr as compared to the contralateral saline-treated control muscles. A similar protective effect of AdV

administration was observed for the decline in dP/dt induced by high-stress eccentric contractions, which was significantly lessened by dystrophin as well as utrophin gene transfer. Importantly, the degree of protection against contraction-induced mechanical injury afforded by AdV-Dys and AdV-Utr did not differ significantly in neonatally-treated mdx animals.

II. Comparative Effects of AdV-Dys and AdV-Utr in Immunocompetent (Adult-Injected) Dystrophic Mice

Maintenance of transgene expression

Photomicrographs representing the different time points examined after AdV-Dys administration to adult mdx mice are shown in Fig. 6, while the corresponding group mean values for dystrophin-positive myofibers are depicted in Fig. 7A. At 10 days after AdV-Dys delivery, myofibers expressing dystrophin averaged 80 $\pm$ 4 (13 $\pm$ 2% of total myofiber number) within the AdV-Dys-injected muscles as compared to 3 $\pm$ 1 (0.5 $\pm$ 0.2%) in untreated control muscles. At 60 days after AdV-Dys administration, there was a marked reduction in dystrophin-expressing fibers to levels that were equivalent to those of saline-injected control muscles. These results are consistent with prior studies of AdV-Dys delivery to adult mdx mice, in which a rapid loss of transgene expression was observed in the absence of immunosuppressive therapy (1;11).

By contrast, the magnitude and duration of AdV-mediated therapeutic gene expression were substantially higher with the use of the utrophin transgene, as shown by the photomicrographs in Fig. 6 and corresponding group mean data in Fig. 7B. Thus, at 10 days after AdV-Utr injection, utrophin-overexpressing myofibers amounted to 387 $\pm$ 43 (52 $\pm$ 3% of total myofibers within the muscle). Although utrophin-overexpressing fibers were reduced to an average of 233 $\pm$ 34 (28 $\pm$ 3% of total

myofibers) by 60 days after AdV-Utr administration, transgene persistence was nonetheless significantly greater than that obtained following AdV-Dys delivery for each time point examined.

In order to determine whether the observed differences in transgene persistence were correlated with the magnitude of cellular inflammation, quantitative assessment of cellular infiltration of muscles in each group of animals was performed as shown in Fig. 8. It should be noted that a background level of inflammatory cell infiltration is normally present in mdx muscles (2). At 10 days after AdV administration to adult mdx mice, the magnitude of inflammation was increased in AdV-Dys-injected muscles in comparison to the baseline level of inflammation found in contralateral control muscles. Importantly, the inflammatory response was significantly lower after AdV-Utr than following AdV-Dys delivery, which is in keeping with the greater persistence of therapeutic transgene expression found in the former group. By 60 days post-AdV administration, the overall level of inflammation was diminished in all experimental groups, with no significant differences being present among groups at this time point.

Dystrophic histopathology

The percentage of centrally nucleated fibers within AdV-Dys- and AdV-Utr-injected soleus muscles of adult mdx mice at 10 or 60 days following vector delivery is shown in Table 1. Since adult mdx animals had already undergone episodes of necrosis and regeneration at the time AdV was administered, central nucleation was present within a substantial proportion of myofibers at the time of initial gene transfer. This is reflected by the high baseline level of central nucleation observed in the non-AdV-injected control muscles of adult animals. At 10 days after AdV injection to adult mdx mice, there was no significant effect of either AdV-Dys or AdV-Utr on the percentage of centrally nucleated myofibers. Furthermore, central nucleation was unaffected by

AdV-Dys administration when followed out to the 60-day time point. Administration of AdV-Utr was associated with a small but statistically significant reduction in the proportion of central nuclei at 60 days post-injection.

Isometric muscle mechanics

Maximal isometric force values in adult mdx muscles injected with AdV-Dys or AdV-Utr are shown in Fig. 9A. In the case of AdV-Dys-injected muscles, values for Po tended to be diminished in comparison to control muscle values at both time points examined. Although values for Po were not significantly improved at the 10-day time point after AdV-Utr administration, there were significant increases in maximal twitch (data not shown) as well as tetanic force generation in AdV-Utr-injected muscles at the 60-day time point in comparsion to both AdV-Dys-injected and untreated muscles. Furthermore, the improvement in tetanic force generation at 60 days after AdV-Utr administration was observed over the entire range of the force-frequency relationship, as illustrated in Fig. 9B.

Susceptibility to high-stress contractions

At 10 days after AdV administration there were no significant effects on the response to eccentric contractions in AdV-Dys- or AdV-Utr-injected muscles, and at 60 days post-injection neither AdV-Dys nor AdV-Utr had any significant effect on the decline in dP/dt (data not shown). On the other hand, a protective effect of AdV-Utr against contraction-induced stress in adult mdx muscles is supported by the fact that the decline in Po at 60 days post-AdV delivery was significantly less pronounced in AdV-Utr-injected muscles than in contralateral saline-injected control muscles from the same animals (see Fig. 10). In contrast, no such protective effect was observed at the same time point following AdV-Dys administration in adult mdx animals.

Table 1. Effect of therapeutic gene transfer on the percentage of centrally nucleated fibers in mdx mouse hindlimb muscles. The percentage of myofibers with centrally located nuclei is shown for AdV-treated and contralateral untreated control muscles. In neonatally (3-5 days old)-injected tibialis anterior muscles, the analysis was performed at 30 days after delivery of AdV-Dys (n=7) or AdV-Utr (n=8); the mean number of fibers counted/muscle = 1141. In adult (45-60 days old)-injected soleus muscles, the analysis was performed at 10 days (n=6 per group) or 60 days (n=5 per group) after AdV administration; the mean number of fibers counted/muscle = 647. *$p<0.05$ vs contralateral saline-injected control muscles.

	Dystrophin		***Utrophin***	
	Control	AdV-Dys	Control	AdV-Utr
Neonatal	43.1±3.1	21.7±4.7*	42.5±2.2	23.6±2.6*
Adult				
10days	42.7±2.8	46.3±2.4	44.3±3.5	46.4±2.5
60days	62.4±1.7	61.1±2.1	60.9±3.2	55.9±3.8*

Figure 1. Dystrophin and utrophin expression after AdV-mediated gene transfer to neonatal mdx muscles. Representative photomicrographs (magnification x200) of immunohistochemical staining for dystrophin (A & B) and utrophin (C & D) in transverse cryosections of neonatal mdx hindlimb (tibialis anterior) muscles examined at 30 days after saline (A & C) or AdV (B & D) injection. Note the large number of myofibers with strong circumferential sarcolemmal staining for either dystrophin or utrophin after AdV delivery, as well as occasional fibers with superimposed cytoplasmic reactivity indicative of marked transgene overexpression. Also note occasional regenerating myofibers in saline-injected mdx muscles with endogeneous utrophin overexpression (arrow in C).

Figure 2. Quantitation of dystrophin and utrophin expression after AdV administration to neonatal mdx mice. The total number of dystrophin- or utrophin-overexpressing myofibers on the entire tibialis anterior muscle cross-section was determined 30 days after saline injection (CON) or adenovirus vector (AdV) injection. Data are mean values $\pm$ SE. Note the absence of any significant differences between the AdV-Dys- and AdV-Utr-injected muscles. *$p<0.05$ vs contralateral saline-injected control (CON) muscles.

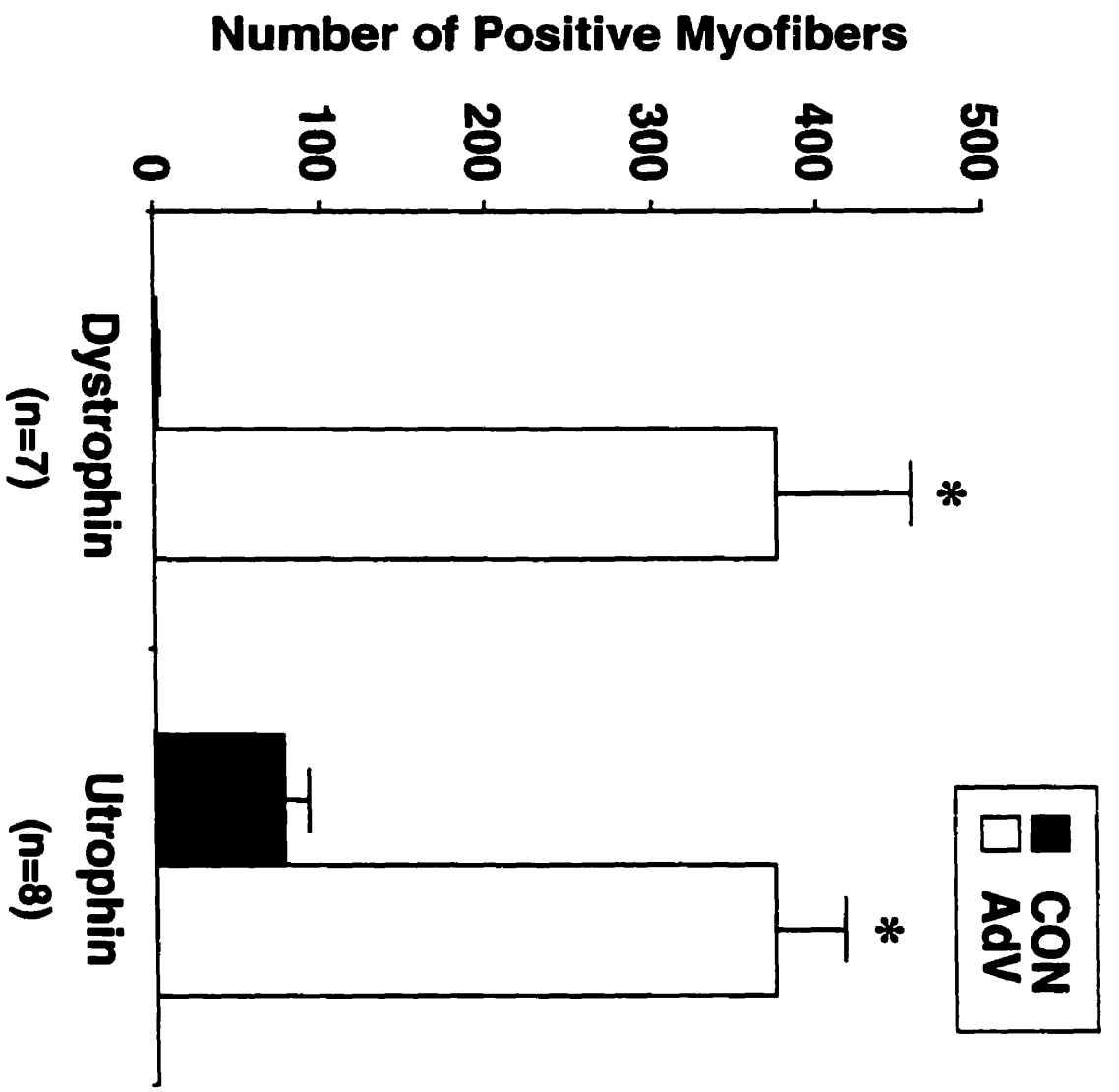

Number of Positive Myofibers
0
100
200
300
400
500
Dystrophin
(n=7)
Utrophin
(n=8)
*
*
CON
AdV

Figure 3. Maximal isometric force production after AdV administration to neonatal mdx animals. Group mean values ± SE for maximal isometric twitch and tetanic force generation at 30 days after AdV-mediated gene transfer are shown. Data are expressed as a percentage of contralateral saline-injected control muscle values (represented by the dashed line) obtained in the same animal. Maximal tetanic force generation was significantly improved by AdV-Dys administration, while a similar trend ($p=0.15$) was also observed in AdV-Utr-treated muscles. There were no significant differences between the AdV-Dys- and AdV-Utr-injected muscles. *$p<0.05$ vs contralateral saline-injected control muscles.

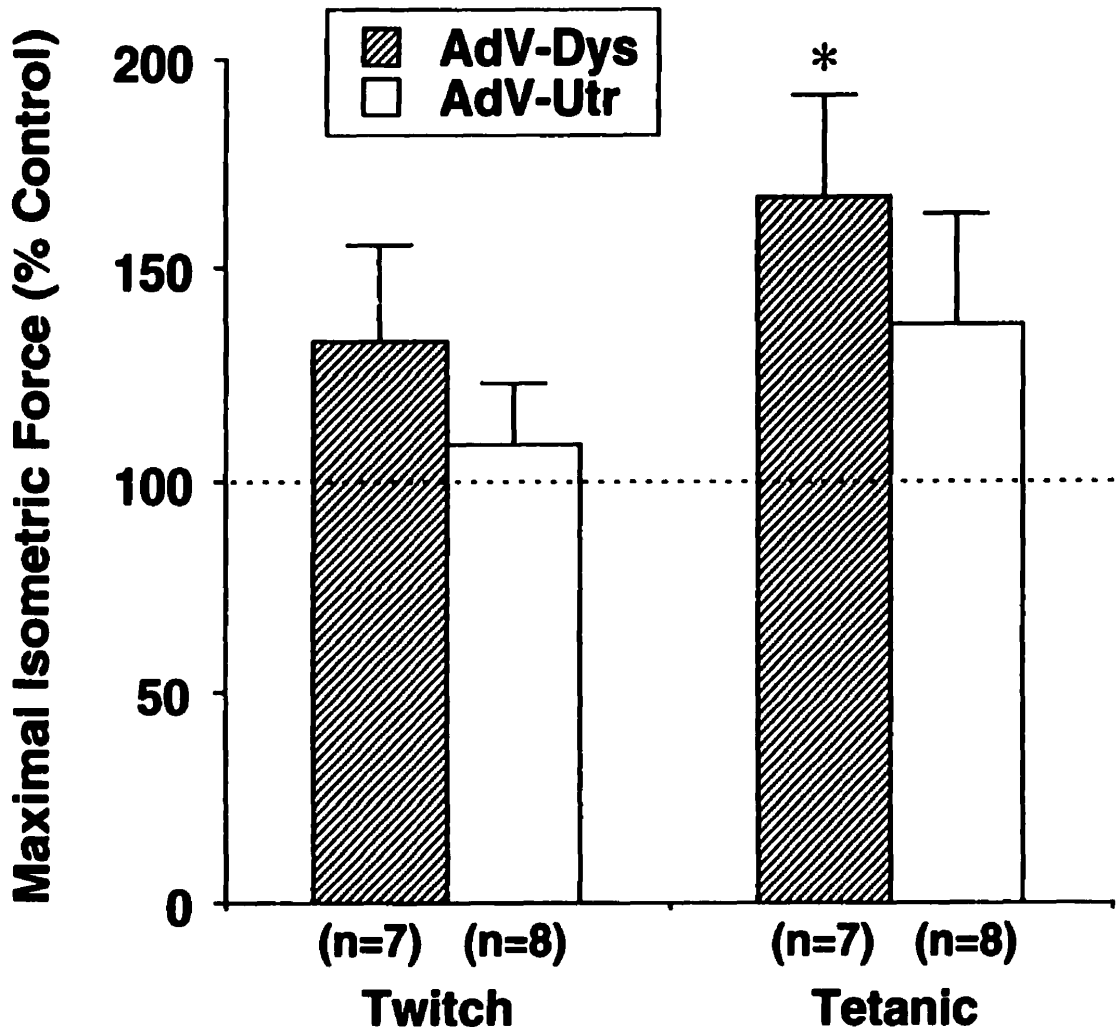

AdV-Dys
AdV-Utr
Maximal Isometric Force (% Control)
200
150
100
50
0
*
(n=7)
(n=8)
(n=7)
(n=8)
Twitch
Tetanic

Figure 4. Schematic representation of physiological signals obtained during the imposition of increased myofiber mechanical stress via eccentric contractions. Simultaneous tracings of length, electrical stimulation and force are shown. The muscle is first stimulated supramaximally and held at its starting length (isometric contraction) in order to measure the maximal rate (dP/dt) and final level (Po) of tetanic force development. Once a plateau in maximal tetanic force has been achieved, the muscle is then forcibly lengthened while electrical stimulation is continued (eccentric contraction). This results in a large increase in the amount of mechanical stress placed on the muscle, which is represented by both peak force (P_{PEAK}) and the force-time integral ($\int P_{ECC} dt$ = shaded area) attained following the eccentric portion of the contraction.

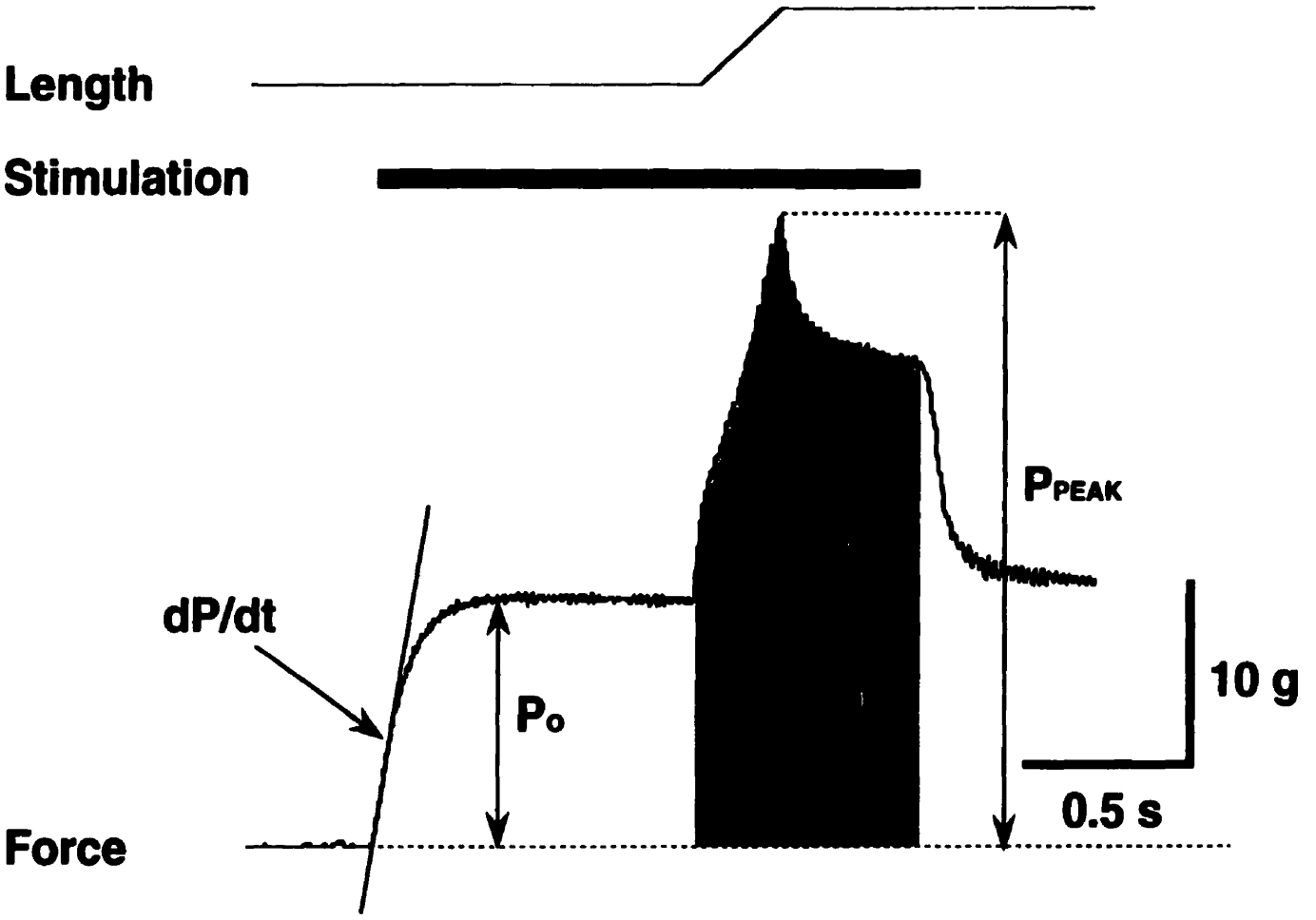
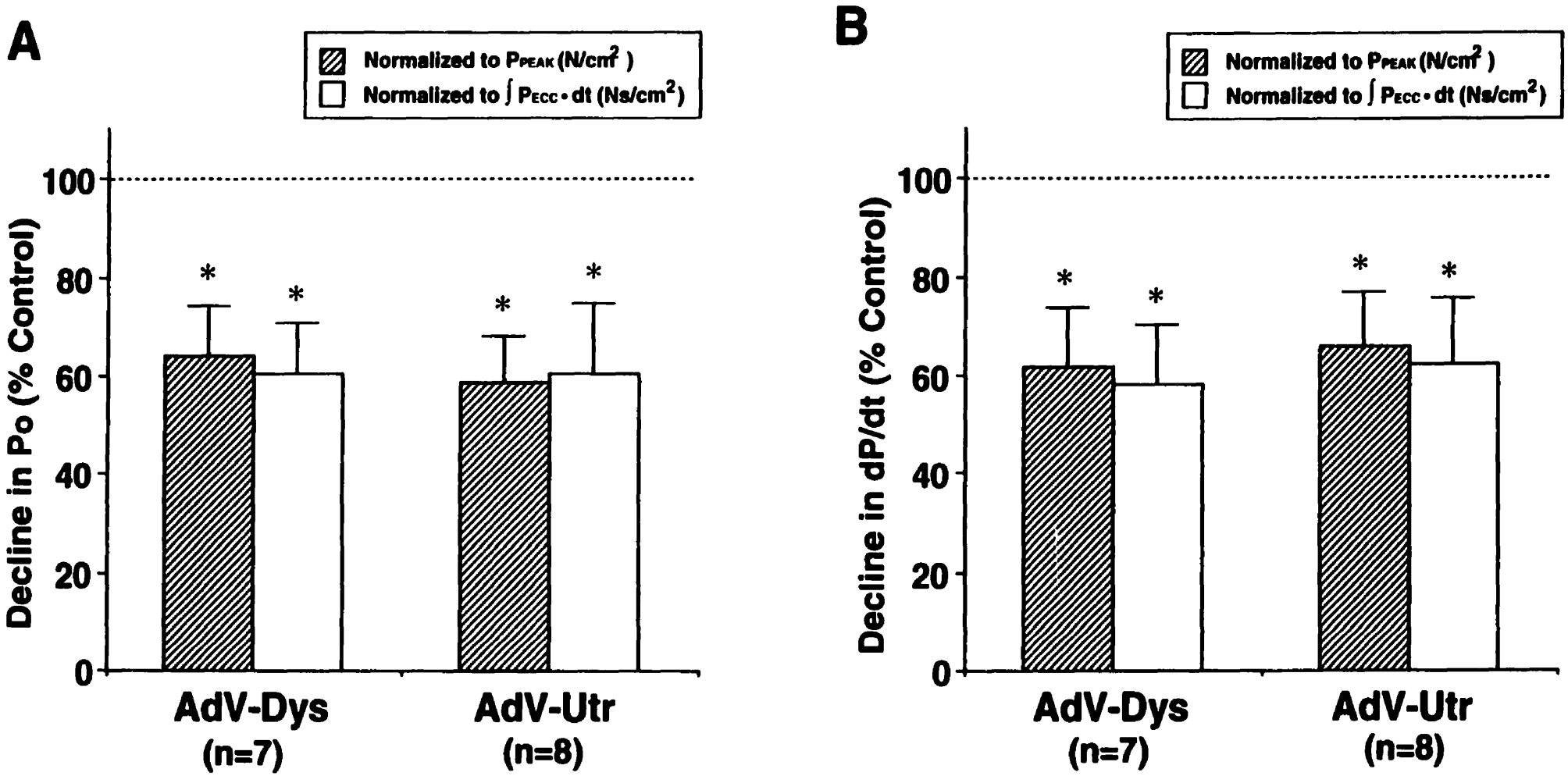

Length
Stimulation
PPEAK
dP/dt
Po
10 g
0.5 s
Force

Figure 5. AdV-mediated dystrophin and utrophin gene transfer afford equal protection against contraction-induced mechanical stress in neonatal mdx mice. Values are normalized to the magnitude of mechanical stress (P_{PEAK} and $\int P_{ECC}dt$) placed on the muscle and expressed as a percentage of contralateral saline-injected control muscle values (represented by the dashed lines) obtained in the same animal. For AdV-Dys- and AdV-Utr-treated muscles, the imposition of high-stress eccentric contractions led to equivalent functional injury. This is indicated by similar reductions in both maximal tetanic force (Po; shown in A) and the rate of force development (dP/dt; shown in B) following eccentric contractions. However, both AdV-injected groups showed significantly less functional injury than was observed in the corresponding contralateral saline-treated muscles (dashed lines). *$p<0.05$ vs contralateral saline-injected control muscles.


A
Normalized to PPEAK (N/cm2)
Normalized to ∫ PECC • dt (Ns/cm2)
Decline in Po (% Control)
100
80
60
40
20
0
*
*
*
*
AdV-Dys
(n=7)
AdV-Utr
(n=8)

B
Normalized to PPEAK (N/cm2)
Normalized to ∫ PECC • dt (Ns/cm2)
Decline in dP/dt (% Control)
100
80
60
40
20
0
*
*
*
*
AdV-Dys
(n=7)
AdV-Utr
(n=8)

Figure 6. Transgene expression after AdV-mediated dystrophin and utrophin gene transfer in immunocompetent adult mdx mice. Representative photomicrographs (magnification x200) of immunohistochemical staining for dystrophin (A, C, & E) and utrophin (B, D, & F) in adult mdx hindlimb (soleus) muscles are shown for the following groups: saline-injected control mdx muscles (A & B), 10 days post-AdV injection (C & D), and 60 days post-AdV injection (E & F). As in neonatal mdx mice, occasional regenerating myofibers with endogenous utrophin overexpression were found in control muscles. At 10 days after AdV injection, numerous fibers exhibited strong circumferential sarcolemmal staining $\pm$ cytoplasmic reactivity for dystrophin (C) or utrophin (D). By 60 days post-AdV injection, dystrophin-overexpressing myofibers were essentially eliminated in AdV-Dys-injected muscles (E), whereas there was a major persistence of myofibers with circumferential sarcolemmal staining for utrophin even at 2 months after AdV-Utr administration (F).

Figure 7. Time course of dystrophin and utrophin expression in hindlimb muscles of immunocompetent adult mdx mice. Group mean values $\pm$ SE are shown for the absolute number of dystrophin (A) and utrophin (B) positive myofibers in adult mdx hindlimb (soleus) muscles at 10 and 60 days after AdV-mediated therapeutic gene transfer. The magnitude as well as the duration of therapeutic gene expression were markedly enhanced with the use of AdV-Utr in immunocompetent adult mdx mice. *$p<0.05$ vs contralateral saline-injected control (CON) muscles.

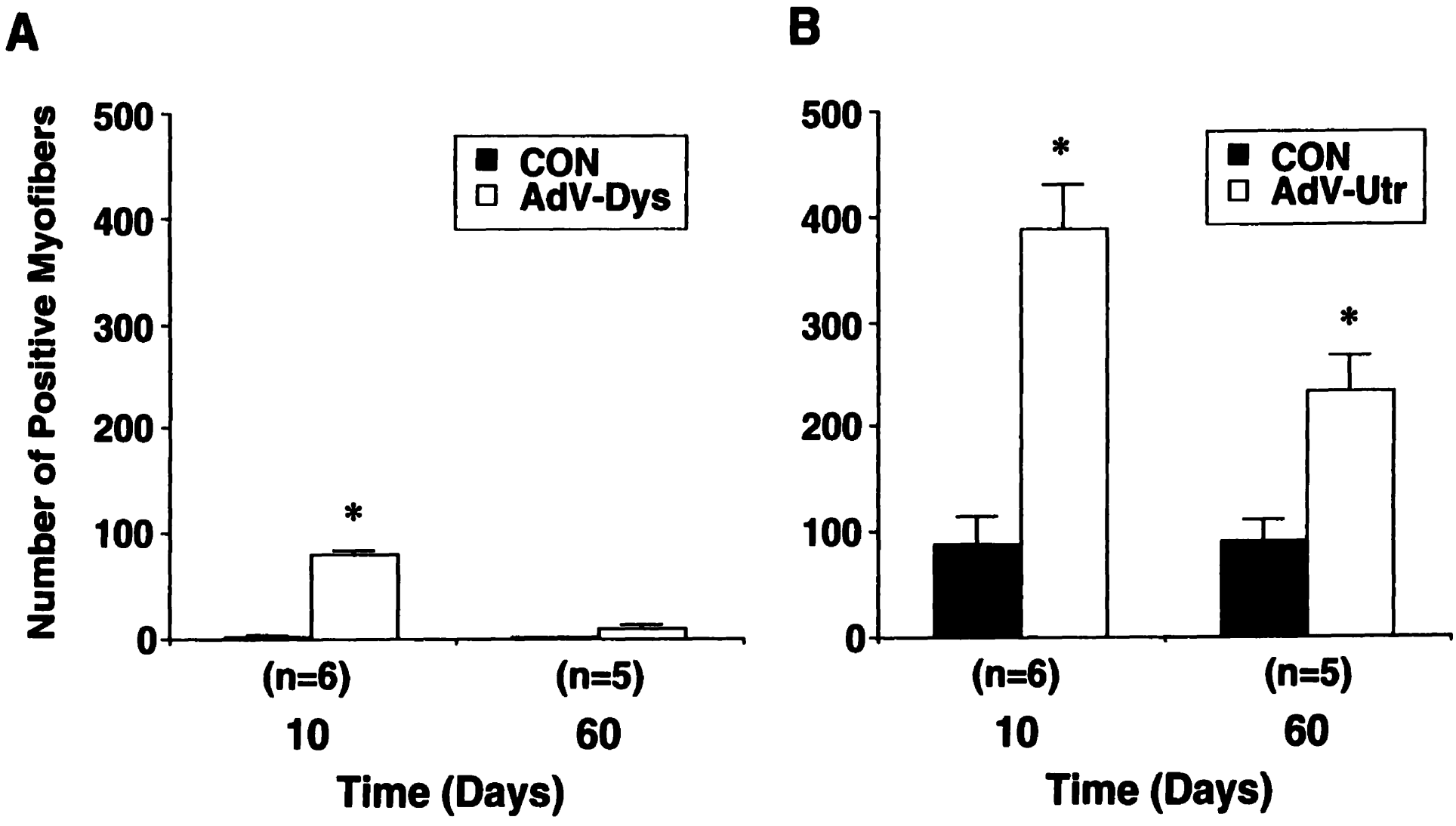

A
Number of Positive Myofibers
500
400
300
200
100
0
CON
AdV-Dys
*
(n=6)
(n=5)
10
60
Time (Days)
B
500
400
300
200
100
0
CON
AdV-Utr
*
*
(n=6)
(n=5)
10
60
Time (Days)

Figure 8. Inflammatory response after AdV-Utr and AdV-Dys delivery to adult mdx muscles. The percent contribution of inflammation to muscle cross-sectional area was compared in AdV-Dys (Dys) and AdV-Utr (Utr) injected adult mdx animals after 10 and 60 days. Note that the inflammatory response was significantly lower after AdV-Utr administration. *$p<0.05$ vs contralateral saline-injected control (CON) muscles; H$p<0.05$ vs AdV-Dys- injected muscles.

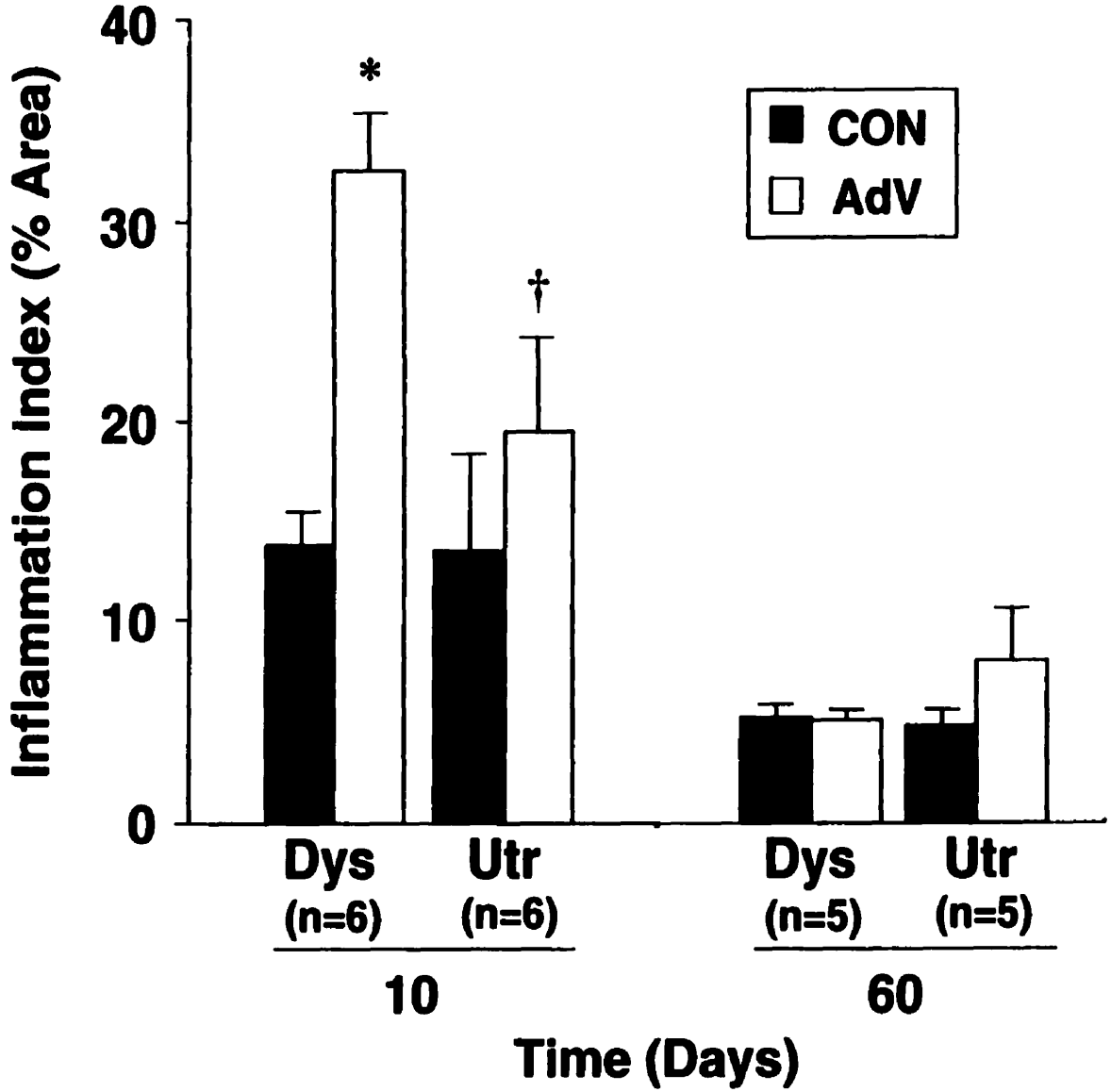
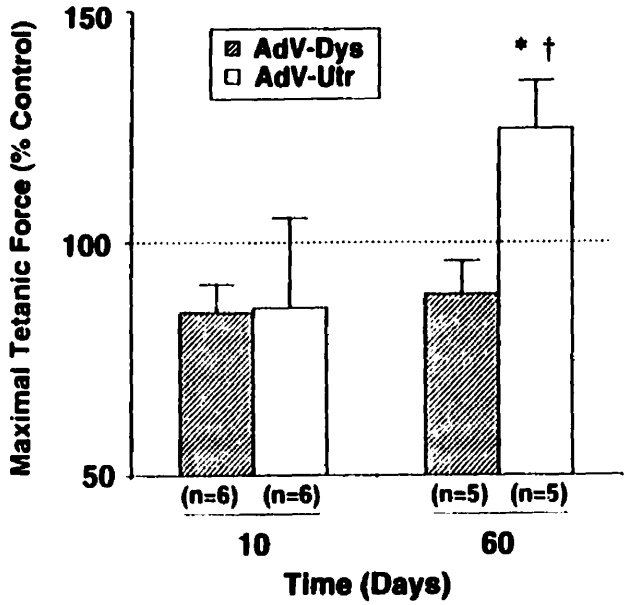

Inflammation Index (% Area)
40
30
20
10
0
*
†
CON
AdV
Dys
(n=6)
Utr
(n=6)
Dys
(n=5)
Utr
(n=5)
10
60
Time (Days)

Figure 9. Maximal tetanic force production after AdV-Dys and AdV-Utr administration to adult mdx mouse muscles. (A) Group mean values ± SE for maximal isometric tetanic force generation at 10 and 60 days after AdV-mediated gene transfer are shown. Data are expressed as a percentage of contralateral saline-injected control muscle values (represented by the dashed line) obtained in the same animal. Force-generating capacity was significantly increased in AdV-Utr-injected muscles at the 60-day time point in comparison to both AdV-Dys-injected and saline-injected control muscles. (B) Force-frequency relationship in AdV-Utr-injected and saline-injected muscles at the 60-day time point (for each group, n=5). Note the significant improvement in force-generating capacity observed across the entire range of physiologic stimulation frequencies in the AdV-Utr-injected group as compared to untreated muscles. *$p<0.05$ vs contralateral saline-injected control muscles; H$p<0.05$ vs AdV-Dys-injected muscles.

A


Maximal Tetanic Force (% Control)
150
100
50
AdV-Dys
AdV-Utr
* †
(n=6) (n=6)
(n=5) (n=5)
10
60
Time (Days)

B

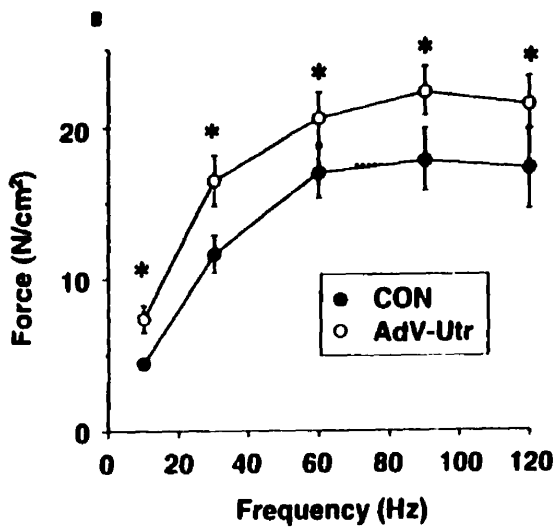
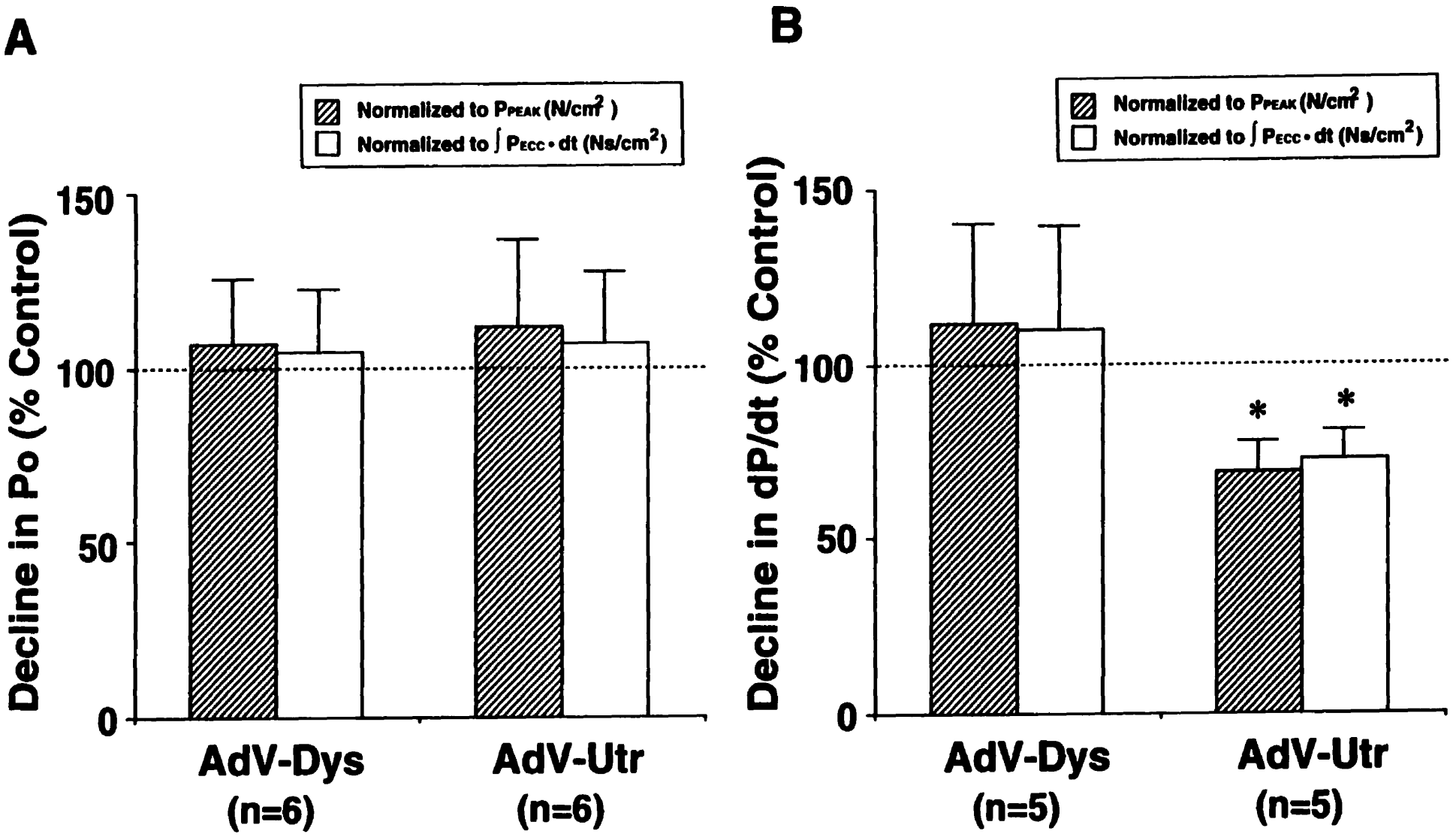

Force (N/cm²)
20
10
0
*
CON
AdV-Utr
0 20 40 60 80 100 120
Frequency (Hz)

Figure 10. Effect of AdV-Dys and AdV-Utr administration to adult mdx muscles on functional injury caused by high-stress eccentric contractions. Values of maximal tetanic force (Po) are normalized to the magnitude of mechanical stress (P_{PEAK} and $\int P_{ECC} dt$) placed on the muscle and expressed as a percentage of contralateral saline-injected control muscle values (represented by the dashed line) obtained in the same animal. At 60 days following AdV-Utr administration, treated muscles showed significantly less functional injury than was observed in the contralateral control muscles (dashed line), whereas no significant difference from control was observed at the same time point in the AdV-Dys-treated muscles.*$p<0.05$ vs contralateral saline-injected control muscles.


A
Normalized to PPEAK (N/cm2)
Normalized to ∫ PECC • dt (Ns/cm2)
Decline in Po (% Control)
150
100
50
0
AdV-Dys
(n=6)
AdV-Utr
(n=6)
B
Normalized to PPEAK (N/cm2)
Normalized to ∫ PECC • dt (Ns/cm2)
Decline in dP/dt (% Control)
150
100
50
0
*
*
AdV-Dys
(n=5)
AdV-Utr
(n=5)

5.6 Discussion

Many characteristics of dystrophic muscle (e.g., reduced force-generating capacity, increased muscle enzyme release into the serum, elevated intracellular calcium, increased connective tissue accumulation) are found to a lesser extent in normal muscles subjected to high-stress muscle contractions (40). There is also evidence that most of the energy lost during cycles of muscle stretch and shortening is absorbed by the sarcolemma (35). Therefore, it has been proposed that muscle disease in DMD patients may represent a pathological amplification of the sarcolemmal damage which occurs in normal myofibers following strenuous muscle activity (31;33). According to this hypothesis, absence of an effective actin-dystrophin-DPC linkage renders muscle fibers abnormally susceptible to contraction-induced sarcolemmal injury, thereby leading to myofiber necrosis unless membrane disruptions can be promptly resealed. In support of the above hypothesis, mdx myofibers demonstrate greater sarcolemmal disruption than normal myofibers after high-stress eccentric contractions, and the degree of such sarcolemmal damage is directly correlated with the level of imposed mechanical stress (31).

This is the first study to directly compare effects of post-natal dystrophin versus utrophin transgene delivery on dystrophic muscle function at different stages of host maturity. We have previously reported that AdV-mediated utrophin gene transfer to immunologically immature neonatal mdx mice leads to a reduction in muscle necrosis and an increased resistance to contraction-induced muscle injury (8;9). Here we expand upon these findings by demonstrating that in neonatally-injected mdx mice, the protective effects of AdV-Dys and AdV-Utr are essentially equivalent. More specifically, reductions in maximum tetanic force generation and the rate of force development normally observed after high-stress eccentric contractions were prevented to a very significant degree by overexpression of either dystrophin or

utrophin, with no measurable difference in efficacy between the two molecules. Furthermore, the central nucleation index, which reflects the level of antecedent muscle necrosis, was similarly and markedly ameliorated when overexpression of either of the two therapeutic transgenes was initiated during the neonatal period. Importantly, the number and distribution of transgene-expressing myofibers did not differ between AdV-Dys- and AdV-Utr-injected muscles, which is in keeping with the fact that the same gene dosage and promoter elements were employed in both groups. Therefore, these findings suggest that in the context of negligible pre-existent muscle damage (24) and the low responsiveness of host cellular immunity (3) found in neonatal animals, there is little or no difference in the therapeutic efficacy of dystrophin and utrophin gene transfer.

However, a very different situation was observed in immunocompetent adult mdx mice, where AdV-mediated utrophin gene transfer was associated with significantly less inflammation and a superior ability to arrest contractile dysfunction than AdV-mediated dystrophin gene delivery. This was reflected by an augmented resistance to contraction-induced mechanical injury as well as a greater preservation of muscle strength in the AdV-Utr-treated muscles. The latter finding is of particular importance, since the primary goal of gene therapy for DMD is to prevent the relentless progression of contractile dysfunction that leads to generalized appendicular muscle weakness and fatal respiratory or cardiac muscle failure. The differences in contractile performance observed between AdV-Dys- and AdV-Utr-treated adult animals can best be explained by the substantially greater persistence of therapeutic transgene expression found in muscles of the utrophin gene transfer group. In addition, the increased muscle inflammation associated with AdV-Dys administration could have directly affected muscle function through local production of force-inhibiting cytokines (41). Taken together, our findings in immunologically mature adult-injected mdx mice

suggest that host immunity against dystrophin could place an important limitation on the therapeutic efficacy of AdV-mediated dystrophin gene transfer in immunocompetent individuals with DMD. In contrast, utrophin gene transfer would appear to be considerably less problematic from this point of view.

In the present study, differences in force-generating capacity between AdV-Utr-treated and untreated mature muscles were observed at 60 days but not at 10 days post-injection, despite the number of utrophin-overexpressing fibers being substantially greater at the earlier time point. It is noteworthy that a similar time course and magnitude of improvement in soleus muscle strength was previously reported after administration of AdV-Dys to immunosuppressed adult mdx mice (44). The time delay required to ascertain the benefit of therapeutic gene transfer is not surprising, since neither dystrophin nor utrophin is directly implicated in the molecular mechanisms underlying force production. For this reason, therapeutic gene transfer would not be expected to acutely restore normal force-generating capacity to adult dystrophic muscles that had already sustained significant functional impairment, nor would it be expected to reverse the presence of central nucleation in previously damaged muscle fibers. Rather, it is anticipated that effective therapeutic gene replacement for dystrophin deficiency will arrest further progression of the disease and thus help to preserve muscle strength over the long term. In this regard, the efficacy of both AdV-Dys and AdV-Utr administration was clearly greatest in immature mdx muscles that had minimal baseline abnormality at the time of AdV delivery.

In previous studies involving transgenic-mdx mice engineered to overexpress the same transgenes employed in this study (32;36), it was difficult to directly compare the therapeutic efficacy of the two molecules for a number of reasons. First, the results obtained for a given transgene construct varied among different transgenic

mouse lines, possibly due to chromosome position effects on the level as well as the distribution of transgene expression within different muscles. Second, promoter elements used to drive transgene expression were not identical for the two therapeutic molecules, which could potentially alter muscle function by differentially affecting expression of endogenous genes of muscle cells via interference with normal *cis-trans* interactions (25). Third, possible differences in functionality between dystrophin and utrophin related to post-natal dystrophic features such as abnormalities of muscle Ca^{2+} homeostasis (42) could not be ascertained, since these abnormalities were essentially preempted by the transgenic-mdx models (7;36). Finally, potential adverse effects of dystrophin or utrophin immunogenicity on therapeutic efficacy could not be evaluated from these studies because the proteins were expressed during embryogenesis with attendant induction of immunologic tolerance.

Early studies of immune responses to AdV attributed diminished transgene expression to adaptive cytotoxic T-cell mediated targeting of viral antigens expressed on AdV-infected cells (46), whereas subsequent work indicated the superimposed immunogenic potential of transgene-encoded proteins (26;37;45). With regard to the latter, previous studies have confirmed the induction of adaptive immunity against either murine (28;39) or human (11) dystrophin introduced into immunocompetent mdx mice. Both cellular (28) and humoral (39) immune responses against murine dystrophin have been reported after transplantation of normal (dystrophin-expressing) histocompatible myoblasts into mdx mice. It is interesting to note that the presence of rare revertant fibers with presumed somatic cell backmutations of the endogenous dystrophin gene, which are found in mdx mice as well as DMD patients, did not confer immunologic tolerance to exogenous dystrophin in these studies. This is likely explained at least in part by missing epitopes within the backmutated dystrophin gene

product (39). Along these same lines, a humoral immune response directed against missing dystrophin epitopes was reported in a human patient who underwent cardiac transplantation with a normal dystrophin-expressing heart (4).

It is likely that the large differences in transgene persistence between AdV-encoded utrophin and dystrophin in immunocompetent mdx mice are explained by the fact that endogenous utrophin expression (found within multiple tissues) conferred a degree of immunologic tolerance to AdV-encoded utrophin. However, a significant reduction in utrophin-overexpressing fibers was nonetheless observed over time, and there are several possible explanations for this phenomenon. First, it is probable that cellular immunity against viral gene products associated with the first-generation adenovirus vector backbone played a significant role in the elimination of myofibers transduced with AdV-Utr (46). A second potential reason for the gradual loss of utrophin-overexpressing fibers observed in our study is inhibition of transcription from the CMV promoter used to drive expression of the transgene (5). This could have been triggered by exposure to cytokines such as interferon-γ(14), which are upregulated in skeletal muscles injected with AdV (38). In addition, use of more elevated AdV dosages has been shown to augment cytokine release (27;34) as well as the rate at which transgene expression is extinguished (26), and a more rapid decline in utrophin expression was also found previously when AdV-Utr was administered at a substantially higher dose (8). Therefore, in the present study the lowest effective AdV dosage was selected with a view toward minimizing this phenomenon as well as dose-dependent acute toxic effects on muscle force-generating capacity that we have documented in previous experiments (44). Lastly, because the utrophin construct utilized in this study contained human cDNA sequences (also the case for dystrophin), a low-grade cellular immune response may have been directed against the utrophin transgene product due to minor species differences in amino acid sequence (37).

However, it is noteworthy that prolonged AdV-mediated expression of the human δ-sarcoglycan gene was recently obtained after skeletal muscle injection in immunocompetent adult hamsters, a finding which was attributed to the high homology among sarcoglycan gene products within and across species (18). The human utrophin and dystrophin constructs used in our expression cassette both demonstrate a similarly high level of homology (91% and 93% identity at the amino acid level, respectively) with the corresponding murine sequence. Nonetheless, it is possible that the advantages of utrophin over dystrophin gene transfer in immunocompetent dystrophic mice documented here would have been further enhanced by the use of a murine utrophin transgene.

In conclusion, the findings of the present study indicate that the therapeutic benefits derived from AdV-mediated gene transfer with either utrophin or dystrophin are largely equivalent when initiated in immature dystrophic animals. In contrast, our data strongly suggest that in the presence of a fully competent immune system, the margin of efficacy as well as safety will be significantly greater when using utrophin rather than dystrophin as a therapeutic transgene for the treatment of dystrophin deficiency. These findings have important implications for future gene therapy efforts in DMD, since attempts to develop less immunogenic adenoviral vectors by deleting all viral genes from the vector backbone (5;13) will not resolve the problem of transgene immunogenicity, and may therefore be less than completely effective when employed in conjunction with a dystrophin transgene. Similarly, the recent demonstration that bone marrow-derived stem cells can be used to restore dystrophin expression to mdx muscle (12), while promising, could also be subject to the same limitation. On the other hand, use of these new gene delivery modes to attain utrophin rather than dystrophin overexpression in muscle could conceivably allow the goal of sustained therapeutic gene expression in DMD to be achieved without the

need for expensive and potentially toxic adjuvant immunosuppressive drug therapy.

5.7 Acknowledgments

This investigation was supported by grants from the Muscular Dystrophy Association, Inc. (USA), the Muscular Dystrophy Association of Canada, the Medical Research Council of Canada, and the National Research Council of Canada. B.J. Petrof and J. Nalbantoglu are Research Scholars of the Fonds de la Recherche en Sante du Quebec. G. Karpati is a Killiam Professor of Neurology through the Isaac Wallace Killiam Endowment of the Montreal Neurological Institute. S. Ebihara is a Fellow of the Medical Research Council of Canada. G.-H. Guibinga received a Studentship Award from the Montreal Chest Institute. We thank J. Bourdon and S. Prescott for expert technical assistance.

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CHAPTER 6

Conclusions

6.1 Conclusions and Implications

The identification of the molecular basis of Duchenne muscular dystrophy (DMD) as being absence of the cytoskeletal protein dystrophin provides the rationale for gene therapy as possible treatment of this degenerative muscle disease. The objective of gene therapy in DMD is long-term expression of functional dystrophin in muscles. So far, animal models of DMD such as the mdx mouse have been used to evaluate the feasibility gene replacement strategies. The therapeutic strategies have included the delivery of myoblasts (autologous or heterologous), naked plasmid DNA, recombinant retrovirus and finally adenoviral vectors. Concerning the latter, its utilization in skeletal muscles is associated with limitations, such as the lack of infectivity of mature myofibers and the immune reaction against adenoviral particles and the dystrophin transgene product. We have tried to overcome these limitations through modification of the host and the transgene. Thus, we have induced muscle regeneration and host immunosuppression, while on the other hand, we have employed an adenoviral vector carrying a less immunogenic transgene.

Through the aforementioned approaches, we have attempted to optimize the expression of the therapeutic transgenes, dystrophin or utrophin, provided by recombinant adenovirus vectors. Thus, in the present thesis, we are the first to have reported the functional risk and benefit associated with injection of myonecrotic substances in dystrophic muscles in advanced disease (Chapters 2 & 3). In the context of DMD, the disease is most of the time brought to medical attention when there are already established signs of the dystrophic phenotype. This stage is characterized by a relative lack of muscle regeneration and consequently the scarce presence of immature AdV-permissive muscle cells. The administration in skeletal muscle of myo-regenerative substances such as notexin that help skeletal muscle to recapitulate the myogenic process could be useful in this setting. It could provide a method for increasing the pool of immature

myofibers and thereby fulfill a major requirement for adenoviral infection of muscle. Alternative methodologies, such as the design of tropism-modified adenoviral vectors, have also been used to increase the tropism of adenoviral vectors for specific tissues (1). The approach consists of engineering a genetic modification of the fiber knob that normally mediates the attachment to CAR (1). However, although successes have been reported using such modified vectors in vitro (10), the bypassing of CAR in this way does not appear to allow efficient gene transfer to muscle in vivo (9).

It is encouraging that we have been able to experimentally induce muscle regeneration in old mdx mice with pre-existent advanced pathology without incurring additional functional impairment. Nevertheless, the methodology and the findings highlighted in Chapters 2 & 3 must be analyzed in the context of their potential application in human dystrophic muscles. We are presuming that our strategy is likely to be successful only if performed in muscles that have not completely exhausted their regenerative potential. Furthermore, even if one can clearly identify the optimal time in disease progression to fully take advantage of muscle regeneration, our strategy still faces major problems. Because of the number of muscles involved by the disease, it will be difficult from a practical standpoint to target all muscles. However, it may be possible to use the paradigm of serial induction of regeneration followed by AdV-mediated gene transfer to treat critical muscle groups such as diaphragm and other respiratory muscles. With respect to the diaphragm, this could conceivably be performed through relatively non-invasive surgery using laparoscopic visualization.

A major limitation of so-called first generation recombinant adenovirus for the treatment of genetic diseases such as DMD has been the short duration of transgene expression due to the host immune response. Previous studies in liver, lung and muscles have shown that the expression of the transgene product as well as viral proteins activates the CD8+ T cells that eliminate transduced cells

(11). Adenovirus vector administration is also associated with activation of B cells that secrete neutralizing antibodies which diminish the efficiency of gene transfer upon repeated administration.

Thus far, a number of immunosuppressive approaches had been employed to increase the duration of transgene after AdV-mediated gene transfer. These approaches include administration of antibodies against CD4+ T cells and CD40, and inducing CD8+ T-cell deficiency (4-6). A group has also reported the induction of antigen tolerance by intrathymic injection of adenovirus (3). However, even if this latter approach is interesting from an academic standpoint, it may have limited practical use in the clinic, since it would likely eliminate the host's ability to fight wild-type adenovirus infection. It has also been argued that vector modification would be preferable to host immunosuppression. The so-called helper-dependent or gutless vectors, in which all of the adenoviral genes are removed and replaced with the gene of interest, have demonstrated a long-lasting expression when administered in liver (2). However, the gutless adenoviral vector will still generate a humoral immune response against viral capsid proteins that can interfere with readministration of the vector.

In Chapter 4 of the present thesis, we have administered a combinatorial immunosuppressive regimen that allows us to tackle the above-mentioned immune-related hindrance. By targeting conjointly T cell receptor-mediated signaling and the CD28-mediated costimulatory pathway through the utilization of FK506 and CTLA4Ig, respectively, we have been able to facilitate both primary and secondary transduction after AdV transduction. Furthermore, in the context of dystrophin gene therapy the transgene constitutes a neo-antigen that ultimately, regardless of the extent of modification that could be accomplished in the vector, will elicit an immune reaction. Our report that the combined utilization of FK506 and CTLA4Ig decreases the immune response against vector

proteins as well as dystrophin is importantly relevant in the context of AdV-Dys gene therapy for DMD.

With further regard to the immune response against the dystrophin transgene product, in Chapter 5 we have evaluated and compared the functional effects of AdV-mediated utrophin and dystrophin gene transfer in dystrophin-deficient muscles of immunocompetent adult mdx mice as well as neonatal mdx mice with an immature immune system. The utilization of utrophin as a surrogate molecule for dystrophin could constitute a promising avenue for DMD gene therapy, since its presence in multiple tissues (including muscle) of DMD patients means that it does not represent a neo-antigen. Although previous studies in transgenic mdx mice showed that overexpression of utrophin could prevent the disease phenotypeb(7;8), we show for the first time that utrophin is actually superior to dystrophin when newly introduced into an adult dystrophic animal with a fully functional immune response. This appears to be related to reduced inflammation and greater persistence of the transgene product with utrophin. These findings suggest that if one intends to use improved gutless vectors to minimize adverse host immune responses, incorporation of utrophin rather than dystrophin into the design of such vectors may give additional benefit from this point of view.

In conclusion, in the present thesis we have improved the delivery of therapeutic genes in a dystrophin-deficient murine model of DMD through modification of either the host or the vector design. Although gene transfer in muscle is still facing major obstacles, the contribution that these studies have brought to the current state of the knowledge represents a significant step in the right direction. We are, however, fully aware of the gap that remains between the encouraging results that we have reported and their full application for a cure of DMD. Our findings may help to provide the foundation for future progress in this area.

6.2 Claims of originality

The work presented in this thesis has provided several original contributions to the existing body of scientific knowledge, these are as follows:

1) Demonstration of the more effective regenerative capacity of dystrophic diaphragm comparatively to normal diaphragm after experimental induction of regeneration with a myonecrotic substance.

2) Analysis of the risk: benefit ratio (found to be favorable) associated with the experimental induction of regeneration prior to the delivery of a therapeutic dystrophin gene by adenovirus vectors in aging dystrophic mouse muscles.

3) Demonstration that blockade of calcineurin through the utilization of FK506, combined with interference of the costimulation signal interaction (CD28:B7) with CTLA4Ig, synergistically act to blunt the immune response against adenoviral antigens and the therapeutic transgene product(dystrophin) in mdx mice. Primary as well as secondary delivery of the therapeutic gene by adenovirus vectors was facilitated.

4) The first comparative study of the therapeutic efficacy of dystrophin and utrophin at different stages of host maturity (adult and neonatal) in mdx mice. We found equivalent therapeutic efficacy for dystrophin and utrophin in neonatal animals, whereas utrophin had a superior therapeutic impact in adult immunocompetent animals.

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