Differential effects of dystrophin and utrophin gene transfer in immunocompetent muscular dystrophy (mdx) mice

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Ebihara, Satoru, Ghiabe-Henri Guibinga, Renald Gilbert, Josephine Nalbantoglu, Bernard Massie, George Karpati, and Basil J. Petrof. Differential effects of dystrophin and utrophin gene transfer in immunocompetent muscular dystrophy (mdx) mice. Physiol Genomics 3: 133–144, 2000.—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 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.

Duchenne/Becker muscular dystrophy; gene therapy; viral vectors; host immunity; muscle mechanics

Duchenne muscular dystrophy (DMD) is the most common X-linked fatal disorder in humans. 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
“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, nonimmunogenic therapeutic transgene.

Utrophin, the autosomal encoded homolog 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 NH2 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 COOH 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 myocardium (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 “self” 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 pathological 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 compared with 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, to more directly assess the relative merits of dystrophin vs. 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.

MATERIALS 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 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 numbers 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). All mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg) by intramuscular 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 $1.5 \times 10^9$ 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 $3.5 \times 10^9$); the contralateral muscle was then again injected with saline alone to serve as a control. The neonatally injected mice were euthanized at 30 days postinjection 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 solution (composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 20 mM NaHCO$_3$, and 6 µM D-tubocurarine chloride) continuously perfused with 95% O$_2$-5% CO$_2$ (pH 7.4) and maintained at 25°C; a thermostabilization 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, Tokyo, Ontario, 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, Montreal, Quebec, Canada) via an analog-to-digital converter at a sampling rate of 1,000 Hz for later analysis. After adjusting each muscle to optimal length ($L_o$, 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 ($P_o$). The muscles were then sequentially stimulated at frequencies of 30, 60, 90, and 120 Hz for 1 s each, with 2 min between each contraction; this allowed maximum isometric tetanic force ($P_{max}$) to be determined. Muscles were then removed from the bath, and $L_o$ was directly measured under a dissecting microscope with microlimiters 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 Newtons per square centimeter.

**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 1,700 ms; the muscle was held at $L_o$ (isometric) during initial 1,000 ms, lengthened through a distance of 5% (for neonatally injected muscles) or 15% (for adult injected muscles) $L_o$ 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 $L_o$. The mean peak force ($P_{peak}$) and force-time integral attained during the eccentric portion of the contraction ($P_{ECC}$;dt) were measured and used as indices of mechanical stress imposed upon the muscles (40). The percent declines in maximal isometric force ($P_o$) and maximum rate of force development (dP/dt) from the first to the last contraction (measured from the isometric component at $L_o$) 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 precooled 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 antidiastrophin (COOH terminus) primary antibody as previously described (1). Utrophin immunostaining was accomplished by blocking with affinity-purified goat anti-mouse IgG (9). Muscle sections were also counterstained with hematoxylin-eosin 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 sarcocellular 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.

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, three to four 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 abnor-
mal 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 Bonferroni correction was applied. All values are means ± SE. Statistical significance was defined as \( P < 0.05 \).

**RESULTS**

**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, whereas the corresponding group mean quantitation for total numbers of dystrophin- and utrophin-expressing myofibers is depicted in Fig. 2. Myofibers expressing dystrophin averaged 375 ± 81 (30 ± 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 myo-

fibers (averaging 0.2 ± 0.1% of total fiber number); these are so-called “revertant” fibers previously noted in mdx mice, which are presumed to represent somatic cell back-mutations of the endogenous dystrophin gene (17). In AdV-Utr-injected muscles, utrophin-overex-

![Fig. 1. Dystrophin and utrophin expression after AdV-mediated gene transfer to neonatal mdx muscles. Representative photomicrographs (magnification ×140) of immunohistochemical staining for dystrophin (A and B) and utrophin (C and D) in transverse cryo-sections of neonatal mdx hindlimb (tibialis anterior) muscles examined 30 days after saline (A and C) or AdV (B and 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 endogenous utrophin overexpression (arrow in C).](http://physiolgenomics.physiology.org)
pressing 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% 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 compared with 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 value. 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 compared with 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 stress-induced injury to muscle is manifested by reductions in both maximal tetanic force ($P_{\text{m}}$) 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 $P_{\text{PEAK}}$ and $fP_{\text{ECC}}dP/dt$) placed on the muscle (see Fig. 4). As shown in Fig. 5, there was a significantly lower decline in $P_{\text{m}}$ in muscles treated with AdV-Dys or AdV-Utr compared with 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.

**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, whereas 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

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**Table 1. Effect of therapeutic gene transfer on the percentage of centrally nucleated fibers in mdx mouse hindlimb muscles**

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<thead>
<tr>
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<th>Dystrophin</th>
<th>Urophin</th>
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<tr>
<td></td>
<td>Control</td>
<td>AdV-Dys</td>
</tr>
<tr>
<td>Neonatal (tibialis anterior)</td>
<td>43 ± 3</td>
<td>22 ± 5*</td>
</tr>
<tr>
<td>Adult (soleus)</td>
<td>43 ± 3</td>
<td>46 ± 2</td>
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<tr>
<td>10 days</td>
<td>62 ± 2</td>
<td>61 ± 2</td>
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<td>60 days</td>
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Values are means ± SE. The percentage of myofibers with centrally located nuclei is shown for adeno-virus vector (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-dystrophin (AdV-Dys; $n = 7$) or AdV-utrophin (AdV-Utr; $n = 8$); the mean number of fibers counted/muscle = 1,141. 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.
80 ± 4 (13 ± 2% of total myofiber number) within the AdV-Dys-injected muscles compared with 36 ± 1 (0.5 ± 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 ± 43 (52 ± 3% of total myofibers within the muscle). Although utrophin-overexpressing fibers were reduced to an average of 233 ± 34 (28 ± 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.

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 compared with 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.

Fig. 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) to measure the maximal rate (dP/dt) and final level (P_o) 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 ([P_{ecc} \cdot dt = shaded area]) attained following the eccentric portion of the contraction.

Fig. 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} \cdot 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 (P_o, 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.
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

Fig. 7. Time course of dystrophin and utrophin expression in hindlimb muscles of immunocompetent adult mdx mice. Values are group means ± SE for the absolute number of dystrophin-positive (A) and utrophin-positive (B) 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.
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 postinjection.

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 $P_o$ tended to be diminished compared with control muscle values at both time points examined. Although values for $P_o$ 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 comparison 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 postinjection neither AdV-Dys nor AdV-Utr had any significant effect on the decline in $P_o$ (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 $P_o$ 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.

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 that 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 postnatal dystrophin vs. 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 preexistent 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 ma-
ture muscles were observed at 60 days but not at 10 days postinjection, 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 postnatal 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. Indeed, 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 back-mutations 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 back-mutated 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). Last, 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 advan-
tages 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 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 expression 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.

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