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Efficient Utrophin Expression Following Adenovirus Gene Transfer in Dystrophic Muscle

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Utrophin is a homologue of dystrophin, the protein whose absence is responsible for Duchenne muscular dystrophy (DMD). As a first step toward clarifying if adenovirus (AV)-mediated utrophin transfer is a possible option to treat DMD, we have constructed an AV expressing utrophin (AdCMV-Utr) and studied utrophin expression after intramuscular injection of *mdx* mice, the mouse DMD model. Overexpression of utrophin by AdCMV-Utr was marked and nontoxic. The recombinant utrophin was distributed homogeneously at the surface of the muscle fibers. Its expression was sufficient to restore the normal histochemical pattern of α-sarcoglycan and β-dystroglycan at this site. These two proteins are members of the dystrophin associated protein complex whose distribution is greatly reduced at the surface of the DMD muscle. These data indicate that AV-mediated utrophin transfer is an efficient way of utrophin upregulation in muscle and has the potential of becoming a treatment for DMD.

Utrophin is a homologue of dystrophin, the protein whose absence is responsible for the progressive and fatal muscle-wasting and weakness of Duchenne muscular dystrophy (DMD) (1, 2). In striated muscle utrophin is less abundant than dystrophin. It is localized to the neuromuscular junctions, whereas dystrophin is distributed over the entire surface of the muscle fibers (3–5). Both proteins bind to actin and to the dystophin associated protein complex (DPC), a large oligomeric structure located at the surface of the muscle fibers and composed of several transmembrane and peripheral membrane glycoproteins (6). One of the characteristics of dystrophin deficient muscle, is a dramatic reduction of DPC at the surface of the muscle fibers (7, 8). Recent studies using transgenic mice, have demonstrated that utrophin is functionally analogous to dystrophin, because the dystrophic phenotype normally observed in the *mdx* mice, the mouse DMD model, is absent when their muscles overexpress utrophin (9–11).

A promising strategy to treat DMD is dystrophin gene transfer using replication defective adenovirus (AV), which has proven beneficial when applied to *mdx* mice (12–14). One potential problem with this strategy is the possibility that expression of dystrophin will elicit an immune response that may undermine any beneficial therapeutic effects of the transgene. In support for this is the fact that antibodies against dystrophin were detected as early as 10 days after inoculation of *mdx* mice with AV expressing dystrophin (15). Such a problem would not exist if the muscles were inoculated with an AV expressing utrophin, because this protein is normally synthesized in muscle. As a first step toward clarifying if AV-mediated utrophin transfer is a possible option to treat DMD, we have constructed an AV expressing utrophin and have studied utrophin expression after intramuscular injection of *mdx* mice. We showed that the recombinant utrophin was efficiently expressed on the plasma membrane of the muscle fibers, and that its expression was sufficient to restore the distribution of the DPC at this site.

MATERIAL AND METHODS

Construction of AdCMV-Utr. An E1 + E3 deleted AV expressing utrophin (AdCMV-Utr) was constructed by cloning the cDNA encoding a shortened version of utrophin modeled on the Becker gene (9) into plasmid pAdCMV-dys (13) by swapping the dystrophin cDNA of this plasmid for the utrophin cDNA, thus generating plasmid pAdCMV-Utr. 293A cells were then transfected with a mixture of LipofectAMINE (GIBCO BRL) containing 3 μg of linearized pAdCMV-Utr (ClaI digestion) and 3 μg of the viral DNA of AdCMV-LacZ (16) after ClaI digestion, according to the manufacturer's recommendation. 16 hours later, the medium was removed and replaced

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with Dulbecco's Modified Eagle Media supplemented with 10% calf serum and 1% SeaPlaque agarose (FMC Bio Product). 10 days later, plaques were picked and expanded as described (17). The expression of utrophin was analyzed by immunoblot using a monoclonal antibody specific for the N-terminus of utrophin (Novocastra, NCL-DRP2) and the presence of the utrophin cDNA in the viral genome was determined by slot blot hybridization as described previously (18). After two rounds of plaque purification, AdCMV-Utr was amplified and purified by centrifugation on CsCl gradients according to standard protocol (17). The preparation was free of E1-containing (replication competent) AV as determined by a very sensitive PCR assay (19) using 500 ng of viral DNA of AdCMV-Utr and primers 5′-CCTGTGTCTAGAGAATGC-3′ and 5′-CAAGTTACGCACAGCAG-3′. These two primers are homologous to nucleotides 1333 to 1350 and 1761 to 1745 of wild type human AV type 5 respectively.

In vivo expression of utrophin by AdCMV-Utr. Five day old *mdx* mice were injected intramuscularly into the tibialis anterior with 5 μl of a fresh preparation of AdCMV-Utr in PBS at a titer of 7.0×10^{11} virus particles/ml as described previously (13). Nine days later, the mice were sacrificed by an overdose of pentobarbital, the tibialis anterior was removed and frozen in liquid nitrogen-cooled isopentane. For immunoblot analysis, 50 cryostat sections were resuspended into 100 μl of sample buffer (62 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, 10 μM PMSF, 2.5 U aprotinin/ml, 0.5 μg leupeptin/ml) and boiled for 4 min. The nuclear DNA was sheared by 20 passages through a 22G 1 = 4 needle and the protein concentration was determined using the BCA protein assay (PIERCE). 25 μg of total protein were separated on a 5.5% SDS-PAGE and blotted on a nitrocellulose membrane. Before loading the samples, 5% β-mercaptoethanol was added and the samples were boiled for 1 min. The blots were incubated with a monoclonal antibody against the N-terminus of utrophin (Novocastra, NCL-DRP2), followed by a peroxidase-conjugated affinity purified goat anti-mouse IgG (Fab specific, Sigma). The signal was then visualized by Enhanced Chemiluminescence (ECL, Amersham). For immunocytochemistry, 4 μm thick cryostat sections were cut and dried at room temperature. The endogenous mouse immunoglobulins were blocked by incubation with an affinity purified goat anti-mouse IgG (Fab fragment, Jackson Immuno Research). The sections were then incubated with one of the following primary monoclonal antibodies: utrophin anti-N-terminus (Novocastra, NCL-DRP2), anti-β-dystroglycan (Novocastra, NCL-43DAG), anti-α-sarcoglycan (Novocastra, NCL-50DAG). To visualize utrophin, the sections were subsequently incubated with Cy3-conjugated affinity purified goat anti-mouse IgG ($F(ab')_2$ fragment, Jackson Immuno Research). To visualize the β-dystroglycan and the α-sarcoglycan, the sections were incubated with biotin-conjugated affinity purified goat anti-mouse IgG ($F(ab')_2$ fragment, Caltag Laboratories) followed by Cy3-conjugated streptavidin (Jackson Immuno Research). Pictures were taken with the 25X objective of a Leitz Orthoplan immunofluorescence microscope using a Kodak Ektachrome P1600 color reversal film. The negatives were scanned and analyzed using the software Adobe PhotoShop.

RESULTS

An E1 + E3 deleted AV expressing a truncated version of utrophin modeled on the Becker dystrophin (9), under the control of the cytomegalovirus promoter (CMV), was constructed and called AdCMV-Utr. Five day old *mdx* mice were injected with 3.5×10^9 virus particles in 5 μl buffer into the tibialis anterior. Nine days later, the mice were sacrificed and the expression of utrophin was investigated by immunoblot and cryostat sections. The recombinant utrophin, which was absent in the uninjected muscles, could be distinguished from the endogenous utrophin because of its smaller molecular weight (200 kDa versus 400 kDa) (Fig. 1). It was obvious, by comparing the intensity of the two utrophin bands, that the level of expression of the recombinant utrophin was several fold higher than that of the endogenous protein. The number of transduced fibers was high and could reach up to 95% of the total fiber population at the site of injection. The recombinant utrophin was evenly distributed at the cell surface (Fig. 2a) and was not restricted to the neuromuscular junctions where it is normally located (3, 4) (Fig. 2d). In many muscle fibers, a substantial overexpression of utrophin resulted in strong cytoplasmic immunostaining in addition to the localization at the cell surface; in such fibers no evidence of toxicity was evident which parallels the lack of toxicity associated with dystrophin overexpression after AV-mediated dystrophin gene transfer (12–14).

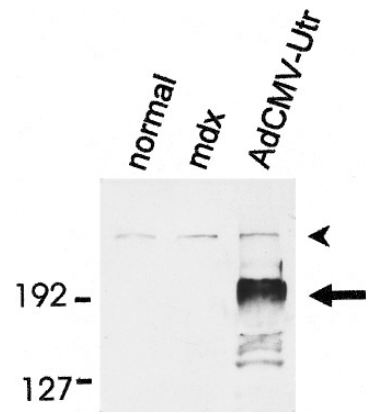

FIG. 1. Abundant expression of utrophin after AV-mediated gene transfer. Muscles of normal, *mdx*, and *mdx* mice injected with AdCMV-Utr were lysed and processed for immunoblot analysis using monoclonal antibodies specific for the N-terminus of utrophin. Arrow: position of the recombinant utrophin present only in the AdCMV-Utr injected muscle. Arrowhead: position of the endogenous utrophin. Numbers on the left indicate positions of molecular weight standard (kDa).

In muscles of DMD patients or of *mdx* mice, the absence of dystrophin results in a dramatic reduction of the DPC in the plasma membrane (7, 8). Serial sections of muscles expressing the recombinant utrophin were stained with monoclonal antibodies specific for β-dystroglycan and α-sarcoglycan, two important components of the DPC (6). In the muscle fibers expressing the recombinant utrophin, a strong signal was observed on the plasma membrane after staining for β-dystroglycan and α-sarcoglycan (Fig. 2b,c) which was absent in uninjected *mdx* muscles (Fig. 2e,f). This indicates that AV-mediated utrophin transfer can restore the distribution of DPC on the plasma membrane.

DISCUSSION

We consider therapeutic gene transfer efficient when it results in significant specific beneficial ef-

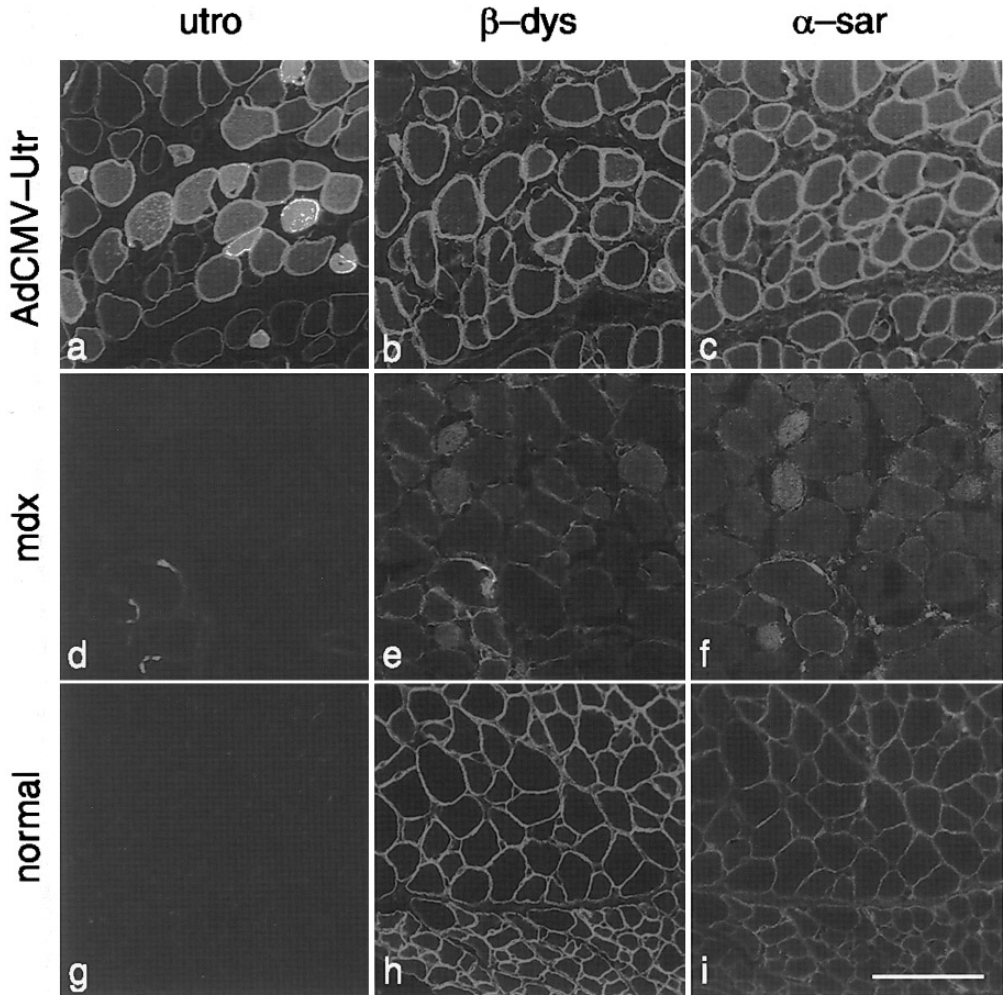

FIG. 2. Overexpression of utrophin restores the distribution of the DPC in the plasma membrane of dystrophic muscle. Consecutive serial sections of muscles of *mdx* mouse injected with AdCMV-Utr (a–c), uninjected *mdx* (d–f), and uninjected normal mice (g–i) were processed simultaneously under the same conditions for immunofluorescence using monoclonal antibodies specific for utrophin (a, d, g), β-dystroglycan (b, e, h), and α-sarcoglycan (c, f, i). In uninjected *mdx* muscle, only the postjunctional regions of muscle fibers show utrophin immunoreactivity (d). Bar = 75 μm.

fect(s) in the tissues where the transgene is expressed. We have demonstrated for the first time the feasibility of expressing significant quantity of utrophin *in vivo* after direct injection of muscles with AV vector. The level of expression of the recombinant utrophin was markedly superior to the endogenous utrophin. It was distributed over the entire surface of the muscle fibers and it was not restricted to the neuromuscular junctions, where it is normally localized (3,4). In many fibers the utrophin excess produced diffuse cytoplasmic immunostaining in addition of the peripheral positivity. No toxicity was noticed as a direct result of utrophin overexpression. One of the main characteristics of the dystrophin deficient muscles is a dramatic reduction of DPC at the surface of the muscle fibers (7,8). We have demonstrated that recombinant utrophin was able to restore the level and distribution at the surface of the muscle fibers of α-sarcoglycan and β-dystroglycan, two important members of the DPC (6), suggesting that utrophin is functionally analogous to dystrophin and can replace it. A similar conclusion was reached when transgenic *mdx* mice overexpressing utrophin were analyzed (9,11). Taken together, these observations support the notion that upregulation of utrophin could be used as treatment for DMD. However, a nontoxic compound that could safely and efficiently upregulate utrophin in muscle, remains elusive. Until such a compound is discovered and its efficiency clinically demonstrated, the most promising treatment for DMD remains AV-mediated dystrophin or utrophin transfer. Several studies have reported that the antigenicity of the transgene is a major factor determining the duration of expression

in *vivo* (20,21,22). Our group has observed that *mdx* mice inoculated with AV expressing dystrophin produced antibodies against this protein (15). The immune response against dystrophin could limit the efficacy of the therapy by eliminating the transduced fibers. Therefore, we think that a therapeutic treatment for DMD based on a utrophin gene transfer strategy could be more advantageous, because utrophin is already present, albeit at low level, in normal and DMD muscle fibers and should not trigger an immune response. In conclusion, the data of the present study suggest that AV-mediated utrophin transfer is an efficient way of utrophin upregulation in muscle and has the potential of becoming a treatment for DMD.

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