Naturally occurring utrophin correlates with disease severity in Duchenne muscular dystrophy

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Although there is good experimental data that utrophin, the autosomal analog of dystrophin, can ameliorate the phenotype in dystrophinopathies, there is scant evidence from human data to support this hypothesis. We investigated in diagnostic muscle biopsies from 16 patients with Duchenne muscular dystrophy (DMD) the level of utrophin expression using quantitative immunoblot analysis. In 13 of 16 patients, in whom there was adequate follow-up data, utrophin expression was correlated to two clinical endpoints: age at reaching Hammersmith score of 30/40 and age at becoming wheelchair-bound. We found that utrophin expression increases with age in DMD and that there is a significant positive correlation between the quantity of utrophin at initial biopsy and time to becoming wheelchair-bound.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal X-linked myopathy due to severe reduction/absence of dystrophin, a subsarcolemmal cytoskeletal protein, which plays an essential scaffolding role in supporting the sarcolemma during contraction in skeletal muscle. Utrophin, of which there are two known full-length isoforms (1), is an autosomal analog of dystrophin and exhibits 80% homology to dystrophin. Data from the mdx mouse, an animal model of DMD, provide evidence that utrophin can functionally compensate and potentially correct muscle pathology due to lack of dystrophin (2–6). Specifically, transgenic mdx mice overexpressing utrophin demonstrate phenotypic and histological amelioration of muscular dystrophy, whereas utrophin knockout mdx mice, lacking both dystrophin and utrophin, develop more severe disease (5,7–12). Furthermore, the extraocular muscles of mdx mice, which are spared of the disease process, exhibit more pronounced upregulation of utrophin, whereas in utrophin knockout mdx mice this sparing is abolished (5). Finally, the dystrophic phenotype of canine X-linked muscular dystrophy is ameliorated by adenovirus-mediated utrophin gene transfer (13).

Data in support of a rescuing role of utrophin in DMD patients are sparse and conflicting. Although upregulation of extrasynaptic utrophin has been demonstrated in DMD and Becker muscular dystrophy (BMD) (14,15), its biological significance has not been proven. There is a report of a single DMD patient exhibiting a severe phenotype with the absence of both dystrophin and utrophin at the sarcolemma (16). There are no published DMD patients with unusually severe phenotypes due to coincidental utrophin gene mutations. Moreover, in DMD, although the extraocular muscles are spared, there is no over and above expression of utrophin in comparison to the rest of the skeletal muscles (17). A previous quantitative western blot study, examining the relationship between utrophin expression and disease severity in DMD and BMD, failed to show any correlation (18). Despite the lack of adequate human evidence to support a potential rescuing role for utrophin in DMD, pharmacological utrophin upregulation is hotly pursued as an alternative therapy to gene transfer for DMD (19–21).

The aim of this study was to evaluate retrospectively the possible disease-modifying role of utrophin in DMD. We first examined the relationship between quantity of utrophin expressed in muscle and the age of the patient at biopsy in 16 cases of DMD. We considered this information essential because not all DMD patients have a biopsy at the same age, and if utrophin expression were age-related, this would have influenced the results of our study. For 13 DMD patients, who had reached pre-specified clinical endpoints, we
correlated clinical data with the amount of utrophin in the muscle biopsy measured by quantitative western blot.

RESULTS

Clinical and molecular findings

All 16 patients underwent routine diagnostic biopsy of the vastus lateralis muscle. Age at muscle biopsy ranged from 7 to 110 months. Age at onset of the disease ranged from 2\(\frac{1}{2}\) to 8\(\frac{1}{2}\) years, but due to variability in early detection of the disease among patients these data were not taken into consideration for further study. Molecular analysis revealed a deletion in the dystrophin gene in nine patients, duplication in two, whereas in five patients neither deletion nor duplication were detected (Table 1). Twelve patients were treated with steroids for an average period of 39.9 months (range 11–83 months). The age, at which a Hammersmith score of 30/40 was reached (in 11/16 patients), indicating moderate disability, ranged from 55 to 126 months (average: 97.5 \(\pm\) 19.5 months). The age at which patients became wheelchair-bound (in 13/16 patients) ranged from 106 to 156 months (average: 134.4 \(\pm\) 13.8 months).

Muscle immunocytochemistry

In all DMD patients, dystrophin was absent from the sarcolemma except in revertant fibers. Utrophin was upregulated along the whole circumference of the sarcolemma, whereas in controls, it was only present along the blood vessels and nerve endings.

Utrophin ratio

Following western blot analysis (Fig. 1), the calculated utrophin ratio (UR) (utrophin to myosin ratio in the patient divided by the utrophin to myosin ratio in the 20-week-old normal fetus expressed as percentage) was obtained in all patients (Table 1). The mean UR in all DMD muscles examined was 55% of the normal fetal muscle with a wide range of 3.6–291% (standard deviation \(\pm\) 70.1%). In comparison, the UR in a normal control muscle was 4.9% of the fetal muscle. The mean UR in dystrophic muscle was 11.2 times of that found in normal adult tissue (range 0.7–59.4). Thus, it appears that there is a significant elevation of the utrophin level in DMD muscle, which in some cases exceeds the level found during the time of physiologically maximal expression in the human fetal muscle, and is on average over 11-fold increased when compared with normal adult muscle.

Relationship of utrophin to age

Spearman’s rank correlation was used to identify the interdependence of patient age at muscle biopsy (\(n = 16\)) and the level of utrophin (UR). This analysis showed that there was a positive correlation between age at biopsy and UR (\(r_s = 0.52; P = 0.019\)). Thus, with increasing age and in spite of disease progression, the amount of utrophin found in surviving muscle fibers in DMD patients increases (Fig. 2A).

Correlation between utrophin level and clinical endpoints

The main purpose of the study was to investigate whether the amount of utrophin expressed in dystrophic muscle has any impact on subsequent disease progression, as measured by two clinical endpoints, age at Hammersmith score of 30/40 and age at wheelchair. Hammersmith score was available in 11 patients and age at wheelchair was available for 13. Spearman’s rank correlation of age at Hammersmith score of 30/40 and UR showed no correlation (\(r_s = -0.06; P = 0.43\)). In contrast, we found a significant positive
correlation between age at wheelchair and UR ($r_s = 0.57$; $P = 0.02$) (Fig. 2B).

As UR correlates significantly with age at muscle biopsy, we performed partial Spearman’s correlation analysis to examine the effect of age at biopsy on the correlation between UR and the two clinical endpoints. This analysis showed that the partial correlation between UR and age at wheelchair was even stronger ($r_s = 0.63$; $P < 0.001$). Similar partial correlation analysis to control for the effect of age at biopsy did not change the lack of correlation between UR and age at Hammersmith 30/40 ($r_s = -0.03$; $P > 0.25$).

Because steroid treatment may also have a significant impact on the progression of DMD (22,23), we performed partial Spearman’s correlation analysis to exclude that the correlation of UR with the clinical endpoints in our patients was in part due to the effect of steroids. After controlling for steroid treatment, partial correlation between UR and age at wheelchair was in fact stronger ($r_s = 0.77$; $P < 0.001$). Similar partial correlation analysis to control for the effect of steroid treatment did not change the lack of correlation between UR and age at Hammersmith 30/40 ($r_s = -0.068$; $P > 0.25$).

**Correlation between steroid treatment and clinical endpoints**

Given the variable duration of steroid treatment in our patients, we examined whether this could affect our clinical endpoints. There was a trend for positive correlation between the duration of steroid treatment and age at Hammersmith 30/40 ($r_s = 0.49$; $P = 0.06$), as well as age at wheelchair ($r_s = 0.44$; $P = 0.06$) but this did not reach statistical significance, perhaps due to the small sample. However, if we control for the effect of UR, partial correlation between steroid treatment and age at Hammersmith 30/40 shows a significant positive correlation ($r_s = 0.49$; $P = 0.025$). The partial correlation of steroid treatment with age at wheelchair becomes stronger and is also statistically significant ($r_s = 0.75$; $P < 0.001$).

In conclusion, our analysis showed that there is a significant positive correlation between utrophin expression in muscle...
and the age at which DMD patients become wheelchair-bound and that this biological effect is independent of the duration of steroid treatment. Therefore, utrophin appears to have an ameliorating effect in these patients and prolongs their ability to ambulate.

**DISCUSSION**

In the current study, we followed-up 16 patients with DMD and attempted to correlate two clinical endpoints, age at Hammersmith score 30/40 and age at wheelchair dependency, to the amount of utrophin in the initial muscle biopsy. Our hypothesis was that if utrophin exerted an ameliorating effect on disease phenotype we might be able to detect this over the range of disease severity observed in DMD. In the subgroup of 13 patients with advanced disease, the age range at becoming wheelchair-bound was 106–156 months, which is comparable to the range of 80–158 months that has been previously reported in untreated patients (24). During the study, medical and paramedical treatment was provided uniformly to all patients at a single center. However, we could not control for other, socioeconomic and psychological factors that could have influenced clinical outcome in our patients.

The mean upregulation in utrophin observed in dystrophic muscle was 11.2 times (range 0.7–59.4) of that found in normal adult tissue. This is similar to what has been previously reported using the Mancho7 antibody (25). At the same time, in our western blots, we used muscle tissue from a 20-week-old fetus as an additional internal control because it has been known that in human skeletal muscle utrophin is maximally expressed between the 18th and 21st week of gestation (26,27). Urophin is subsequently downregulated at the sarcolemma and is only present at the neuromuscular and myotendinous junctions at birth. We found that most DMD patients (14 out of 16) had less utrophin compared with the 20-week-old fetus, with a range of 3.6–290% of the fetus. Thus, compared with the normal adult muscle, utrophin is substantially upregulated in DMD but is well below (average of 55%) the amount present at the period of maximum expression in the fetus.

Our study showed a positive correlation between age at biopsy and level of utrophin in the DMD muscle. This is in agreement with immunocytological studies by Taylor et al. (28) and by our group (29). Although we did not investigate in the current study the mechanism leading to increased utrophin levels with age, this phenomenon is probably due to a time-dependent accumulation of utrophin at the sarcolemma as a result of altered translation (30). The increase of utrophin with age could not be the result of steroid treatment, as this was started after the time of biopsy in all of our patients. Furthermore, it has been shown that utrophin is not upregulated following prednisolone treatment in the mdx mouse (31). There is evidence that transcription of the utrophin gene remains rather constant throughout the life span of a muscle fiber both in normal and DMD muscle (32). Given that both dystrophin and utrophin interact with the dystrophin-associated proteins complex (33), when dystrophin is lost utrophin may occupy some of its bindings sites on β-dystroglycan. This is supported by studies showing reciprocal expression of dystrophin and utrophin in individual muscle fibers from BMD patients and DMD carriers (14,15).

The second finding of the present study was the positive correlation between utrophin expression and age at reaching the wheelchair stage consistent with an ameliorating role for utrophin in DMD. This effect was independent of the duration of steroid treatment and statistically significant. Our findings are in contrast to those by Vainzof et al. (18), who failed to show any correlation between utrophin and disease severity in either DMD or BMD. However, in that study, the effect of utrophin level on disease severity was not controlled for age at biopsy and the authors did not state whether any of their patients were treated with steroids. We were not able to correlate utrophin expression with age at reaching Hammersmith score of 30/40, perhaps due to the fact that the latter is a less robust clinical endpoint requiring the cooperation of the patient. The small number of patients might have also obscured any relationship.

We found a positive correlation between the duration of steroid treatment and the two clinical endpoints which was not statistically significant. This is again perhaps due to the small number of patients studied. However, after controlling for the effect of utrophin level, the partial correlation with both clinical endpoints became statistically significant, in agreement with the existing literature about the beneficial effect of steroids (22,23).

Although the age-dependent gradual accumulation of utrophin at the sarcolemma of DMD muscle fibers does not appear to arrest the disease process, it has a positive effect on disease severity. There is evidence from transgenic mdx mice overexpressing utrophin that the precise timing of increased utrophin expression and the absolute amount of utrophin expressed are critical for ameliorating the phenotype. Urophin overexpression could significantly ameliorate phenotype only if it was upregulated before but not after 30 days of age (7). If the same biological phenomenon occurs in DMD this would perhaps partly negate the beneficial effect of an age-dependent upregulation of utrophin at the sarcolemma of myofibers.

Patient 6 expressed the highest utrophin level (UR: 290% of fetal muscle), well above the levels found in the other patients and raises a number of points. Although utrophin expression increases with age, there are clearly other factors that modulate utrophin expression. His utrophin level was almost three times higher than those seen in a 20-week-old fetus, a point in time at which the utrophin gene is naturally maximally expressed in the human, yet his phenotype was not markedly different from the rest of this group. Thus, there are important factors other than utrophin that modulate phenotype in DMD. A 3–4-fold upregulation of utrophin at the sarcolemma of mdx mouse prevented muscular dystrophy (9). This would have corresponded to a UR of 165–220%, three to four times the mean UR of 55% in our patients. Whether such a level of upregulation of utrophin can ameliorate the DMD phenotype remains to be determined.

Despite some limitations, including the small number of patients, our study demonstrates a significant increase of utrophin with age in DMD muscle and a positive effect of utrophin on disease severity as measured by the age at which the patient...
becomes wheelchair-bound. This is perhaps the most clear cut evidence from human material that utrophin may indeed assume a rescuing role in DMD. Further studies are indicated to determine the degree and timing of utrophin upregulation needed to halt disease progression, as well as the means of achieving such an upregulation to the extent that it can significantly improve outcome in DMD.

MATERIALS AND METHODS

Patients

Sixteen DMD patients were diagnosed at our institution by DNA analysis (34) and by muscle dystrophin and utrophin immunocytochemistry (Table 1). The degree of functional disability was assessed using the Hammersmith score (35). A score of 30/40 indicates moderate disability, whereas a score of 25/30 approaches the wheelchair-bound stage. As clinical endpoints, we considered the time to reach Hammersmith score of 30/40 and the time to reach wheelchair dependency. Thirteen of these patients have reached both clinical endpoints of disease progression (but in only 11/13 was the age at Hammersmith score 30/40 available) and were included in the study of utrophin effect on disease progression. Results of biopsies from three patients, who have not yet reached these clinical endpoints, were analyzed only in regard to the relation between age at biopsy and level of utrophin.

Muscle biopsies

The muscle biopsies were obtained under general anesthesia from the vastus lateralis in all patients. Muscle was snapped frozen in nitrogen-cooled isopentane and stored in −70°C. Histology and histochemistry were carried out using standard methods (36). Dystrophin immunocytochemistry (Novocastra Dys 1, 2, 3) was performed using a double reaction and an immunofluorescence method (29).

Immunoblots

Approximately 15–25 mg of muscle were homogenized with nine volumes of sample buffer [60 mM Tris–HCl buffer, pH 6.8, 10 mM EDTA, 10 mM EGTA, 50 mM dithiothreitol, 10% sodium dodecyl sulfate (SDS), 6% glycerol]. Aliquots were removed from this homogenate for protein determination using a mini Lowry procedure. The homogenate was diluted so as to give a protein concentration of 2.5 mg/ml, boiled for 2 min and centrifuged at 15 000 r.p.m. (20 000g) for 5 min. Twenty microliters of each supernatant were loaded in each well (total protein 50 μg). The muscle proteins were separated on a 6% SDS–polyacrylamide gel (1 mm × 15 cm × 13 cm; 15 lanes) with a 4% stacking gel. Electrophoresis was run at 60 mA (for two gels) in 25 mM Tris–glycine buffer, 0.1% SDS, until the dye front reached the bottom of the gel. The gel was cut above the myosin band (as indicated by the pre-stained standard) and the top part was electroblotted onto a nitrocellulose membrane (Amersham Hybond-C extra) in 25 mM Tris–glycine buffer, 10% methanol, 0.005% SDS, at 0.4 A overnight and with cooling. The membrane was probed for 2 h with the Mancho3 anti-utrophin mouse monoclonal antibody, kindly provided by Professor G.E. Morris (37), diluted 1:100. The secondary antibody was diluted 1000 times and utrophin was visualized using the Amersham chemiluminescence kit (ECL, RPN2108). The membrane was exposed to X-ray film for 15–150 s. The bands were scanned and integrated using an LKB Ultrascan Laser XL Enhanced Scanning Densitometer (Gelscan XL version 2.0 software). The bottom part of the gel was stained in Coomassie blue (1% in 38% ethanol and 0.1% acetic acid) and destained in 12% ethanol and 0.05% acetic acid. The myosin bands were scanned and integrated using the same scanner as above.

Quantification of utrophin

The intensity of the utrophin band was expressed as a ratio to the intensity of the myosin band (myosin on the gel, not transferred to the membrane) to allow for variations due to differences in loading, even though the muscle homogenates were made up to the same protein concentration before loading. In order to control for variation in exposure time of the film, each ratio (utrophin/myosin) was further normalized by expressing it as a percentage of the ratio (utrophin/myosin) of a muscle sample from a normal 20-week-old human fetus run on the same gel and exposed onto the same film. This value is called the UR. Fetal muscle was used as a control because utrophin is maximally expressed in normal humans at about 18–21 weeks of gestation (26,27). Normal adult muscle was also used as a control in every experiment. Each sample was run in triplicate on the same gel and the mean UR taken.

Statistical analysis

We used non-parametric rank-order correlation analysis (Spearman’s rs and partial Spearman’s r coefficient) to assess the relationship between the age at biopsy and UR and between the UR or duration of steroid treatment and the clinical endpoints. Statistical significance was set at P ≤ 0.05 (one-tailed test).

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Conflict of Interest statement. None declared.

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