Laminin-α2 but not -α1-mediated adhesion of human (Duchenne) and murine (*mdx*) dystrophic myotubes is seriously defective

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Abstract It has been suggested that α-dystroglycan links the dystrophin-associated protein complex and extracellular matrix and that the absence of dystrophin and α-dystroglycan in Duchenne muscular dystrophy (DMD) may lead to the breakdown of this linkage. In the present study, myotubes from DMD patients and murine X-linked muscular dystrophic mice (*mdx*) were used to measure their adhesive force to the physiological laminin-α2 substrate, and it was found that the dystrophic myotubes were selectively unable to sustain adhesion. However, normal and dystrophic myotubes attached equally well to the laminin-α1 substrate. As far as we know, this is the first experimental evidence that the absence of dystrophin causes the complete loss of a still unknown laminin-α2-dependent adhesion force, therefore suggesting that the primary consequence of Duchenne dystrophy consists of the loss of an authentic mechanical linkage at the level of the α-dystroglycan/basal lamina interface.

Key words: Duchenne dystrophy; *mdx* myotube; Merosin; Laminin; α-dystroglycan

1. Introduction

Dystrophin is missing in Duchenne dystrophy and the dystrophin-associated protein (DAP) complex is either poorly expressed or totally absent. Sarcoglycans and dystroglycans are major components of the DAP complex, and a number of diseases are due to mutations in the former [1,2]. In muscle, the DAP complex appears to play two important roles: it is involved in the stabilisation of the acetylcholine receptor (AChR) clusters linking agrin and utrophin [3] and it binds merosin, one of the most important proteins of the extracellular matrix (ECM), to the sarcolemma. Both of these functions are achieved by α-dystroglycan (previously known as cranin [4,5]), a 156-kDa protein expressed in muscle, brain and other tissues that binds in a Ca^{2+}-dependent manner to the distal end of the α-chain of laminin via the last two globular repeats [6].

Although it has been hypothesised on structural grounds [1,2] that DAP is the major mechanism of attachment between the cytoskeleton and ECM in skeletal muscle, there are no demonstrations of its quantity or functional role.

In the present paper, we provide evidence (with quantitative measurements [7]) that the forces sustaining the adhesion of DMD and *mdx* myotubes to laminin-α2 are several times less strong than those of normal myotubes, thus supporting the notion that the lack of α-dystroglycan has dramatic consequences on the machinery sustaining adhesion. Moreover, our results highlight the mechanical role of α-dystroglycan when it binds to merosin in ECM and thus links the cytoskeleton to ECM in normal muscle cells.

2. Materials and methods

2.1. Culture

2.1.1. Mouse. The myotubes were prepared from the limb muscles of 3–4-day-old normal (C57BL/10ScSn) and *mdx* (C57BL/MDX) mice following procedures previously described [8]. Briefly, the explants of muscle tissue were plated in 80% HAM F-10 culture media (Sigma), 20% fetal calf serum (FCS, Hyclone, The Netherlands), 2 mM glutamine, 2.5 ng/ml basic fibroblast grown factor (FGF, F-0291, Sigma, Italy) for normal and 0.5 ng/ml for *mdx* myoblasts [9]. In this proliferative medium, myoblasts and satellite cells spread out in 3 days. On the third day, they were replated in a differentiative medium of 98% DMEM (Hyclone) and 2% horse serum (HS), in which the mononucleated muscle cells initiated a terminal differentiation consisting of cellular fusion and myotube maturation. The myotubes were mechanically detached and resuspended in DMEM, 250 μg/ml bovine serum albumine (BSA), 1.74 mM EGTA (external Ca^{2+} was reduced in order to decrease Ca^{2+} influx and prevent contraction), and then plated on laminin-α1 (Collaborative Research, USA, 2 μg/ml) or -α2 (Chemicon, USA, 5 μg/ml) -coated dishes previously saturated with 250 μg/ml BSA (Sigma, Italy). After 12–24 h, the dishes were used for adhesion experiments.

2.1.2. Human. Primary myoblasts were obtained from muscle biopsies of three DMD patients and three age-matched normal controls following procedures previously described [10]. The DMD patients had the clinical and pathological features characteristic of DMD, and the absence of dystrophin was assessed in their muscle by means of immunohistochemistry as previously described [11]. The normal controls were individuals who had undergone muscle biopsy for diagnostic purposes but were found to be free of muscle disease. Briefly, the muscle samples were freed of fat and connective tissue, minced to obtain fragments that were smaller than 1 mm^3, and then dissociated by means of two or three successive treatments with trypsin-EDTA. After dissociation, the cells were washed, resuspended in culture medium (see below), plated in culture dishes and grown to 70% confluence. In order to eliminate fibroblast contamination, the cells were harvested by means of trypsin treatment, washed, incubated with Texas red-conjugated anti-CD56 monoclonal antibody (Becton-Dickinson, San Jose, CA) and sorted using a fluorescence-activated cell sorter (FacStar Plus, Becton-Dickinson) according to the procedure described by Webster et al. [10]. The myoblasts were plated in 60 mm petri dishes and maintained at 37°C and 5% CO_2 in the following medium: DMEM (Gibco, Gaithersburg, MD) containing 20% FCS (Hyclone, Logan, UT), 10 ng/ml epidermal growth factor (EGF, Gibco) and 10 μg/ml bovine insulin (Sigma, St. Louis, MO). In order to obtain myotube cultures, the myoblasts were plated in 35 mm laminin-α1 or -α2-coated dishes and allowed to fuse (4–5 days) into multinucleated myotubes by replacing the growth medium with a fusion medium containing 2% FCS without mitogens.

2.2. Quantitative measurements of adhesion

2.2.1. Procedure. In order to determine precisely the force exerted by the binding of ECM to its receptors, we developed a quantitative method of measurement. Briefly, Petri dishes with primary cultures of mouse myotubes were placed in suitable positions in classical centri-

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fuges (Labofuge M, Hereus) in order to apply forces (proportional to the amount of acceleration and the mass of the myotube) that were perpendicular to the plating surface and break cell adhesion. By varying the speed of the centrifuge during predetermined periods of times (1.5–2 min), the forces could be applied in a reproducible manner. Videotape recording set-up was used to store 144 1×1 mm images, for each petri dish, before and after centrifugation. Using two screens the number of myotubes of corresponding images was counted and we derived the percentage of myotubes remaining attached in the same petri dish (% adhesion, mean ± SEM) before and after the application of increasing forces. The Petri dishes were used only once and, after the treatment, the attached cells were still in perfect physiological condition (normal resting potential and contractility, not shown).

Briefly, the rationale underlying the procedure is as follows. The adhesive force (F_a) is proportional to specific adhesion (α) multiplied by the adhesion area, which is approximately proportional to the product of myotube length (l) and diameter ($2r$), giving $F_a = \alpha \times l \times 2r$. The centrifugal force (F_c) is proportional to the product of acceleration (a) and mass, which is proportional to the myotube length and cross-sectional area (πr^2) giving $F_c = a \times l \times \pi r^2$. A cell detaches when $F_c > F_a$, and so, if all other conditions are equal, the larger myotubes (larger r) detach first. In particular, we expect that % adhesion (as a function of centrifugal acceleration) should follow a sigmoidal curve (see dotted line, Fig. 1, right) that is more or less steep according to the variability in the myotube population: steep for an almost uniform population and less steep for myotubes of very different dimensions. Under these conditions, the half-way point of the curves also becomes a reasonable absolute measure of adhesion force. From our data, we can calculate that the force sustained by a typical myotube (100 μm long, 10 μm wide and 2 μm thick) before becoming detached is roughly 4×10^{-5} dyne, a value that corresponds to a force per surface area of about 4 dyne/cm^2, which is in good agreement with the data reported in the literature [12].

2.3. Test and control experiments

The sensitivity of the method was tested under experimental conditions in which the substrata and extracellular media were changed. The plot of the % adhesion of control murine myotubes to varying concentrations of laminin-α1 is shown in Fig. 1 (left) at one single force (corresponding to an imposed acceleration of 50 g). In order to gain insight into the detection limits of the method we investigated the effect of different concentrations of substrate to varying forces as shown in Fig. 1 (middle), where it is possible to see that the complete removal of the attachment of the myotubes is clearly described at a concentration of 2 μg/ml but not 10 μg/ml of laminin-α1. On the whole, the results of these experiments indicated that, in order to show a correct description of the detachment and to minimize the possibility of cell damage [13,14], we should reduce the force required to detach the cells. Accordingly, we decided to decrease the concentration of substrate to 2 μg/ml in all the successive experiments. This is in agreement with the fact that it is known that α-dystroglycan binds laminin with affinities substantially higher than laminin receptors of the integrin superfamily [6].

In order to verify whether the method was sensitive enough to detect different origins of the adhesion process, we performed experiments in two different extracellular media (Hanks' balanced salt solution, with Ca^{2+} and without Mg^{2+} or vice versa), which should qualitatively correspond to α-dystroglycan-dependent [6] and integrin-dependent adhesion [15–17], respectively. The results of these experiments are shown in Fig. 1 (right), in which it is possible to see that, in laminin-α1, myotubes adhere more strongly under $+Mg^{2+}/-Ca^{2+}$ (Fig. 1, ▼) conditions than in $-Mg^{2+}/+Ca^{2+}$ conditions (Fig. 1, ▲); in the case of laminin-α2 (5 μg/ml; Fig. 1, △,▽) the differences in adhesion do not appear to be significant. On the whole, the results of these control experiments suggest that the procedure for evaluating adhesion is sensitive enough to distinguish the various types of substrata and conditions that mediate the strength of the mechanical adhesion of myotubes to physiological substrata. All of the data presented in the Results section were obtained under $-Mg^{2+}/+Ca^{2+}$ conditions.

2.4. Immunofluorescence of DMD myotubes

For each patient, myotube-containing petri dishes were stained in order to detect any dystrophin-positive myotubes formed from revertant nuclei. The cells were fixed in methanol and dystrophin was localised by means of a polyclonal antibody directed against the fusion peptide located at the C-terminus of the protein using a biotin-avidin system [18]. In the same dish, 4′,6-diamidino-2-phenylindole (DAPI) was used to mark nuclei. Percentage counts of the myotubes (≈200/dish) showed that an average of only 6% of the total number of myotubes were dystrophin-positive.

3. Results

3.1. Myotubes from normal and 'mdx' mice adhere to laminin-α1 equally well

The same procedure as that shown in Fig. 1 was used to

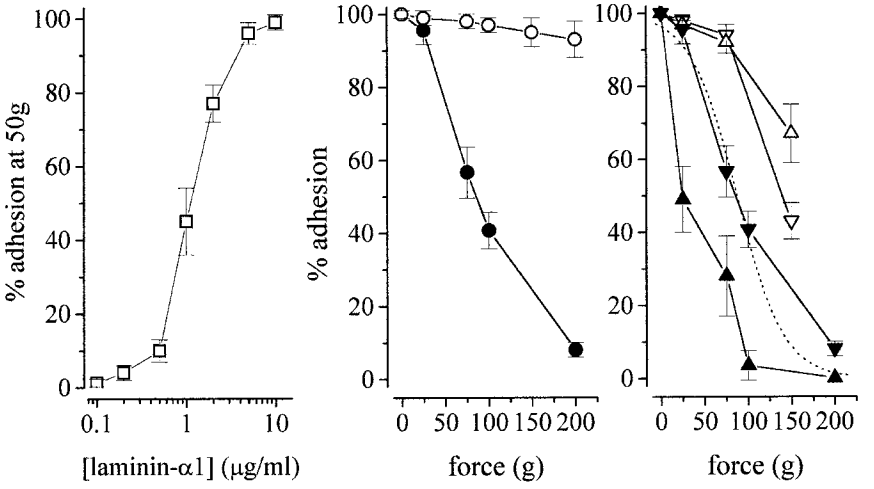

Fig. 1. Characterisation of the methodology (mouse myotubes). Left: Plot of % adhesion ($n=4$) at a fixed force (calibrated in acceleration, g units) of mouse myotubes as a function of laminin α1 concentration. Middle: % Adhesion ($n=4$) as a function of the perpendicular force applied to the petri dish at two different laminin-α1 concentrations of 10 (○) and 2 (●) μg/ml, respectively. Right: Effects of media with different divalent cations. The data derive from experiments using laminin-α1 (▲,▼) or laminin-α2 (△,▽), and in the presence of Mg^{2+} without Ca^{2+} (▽,▼) or in the presence of Ca^{2+} without Mg^{2+} (△,▲); see Section 2. Each point is the mean of four different experiments. The dashed line is the sigmoidal curve which best fitted the experimental points (▼). The average number of counted cells/petri dish (experiment) was 320 ± 21.

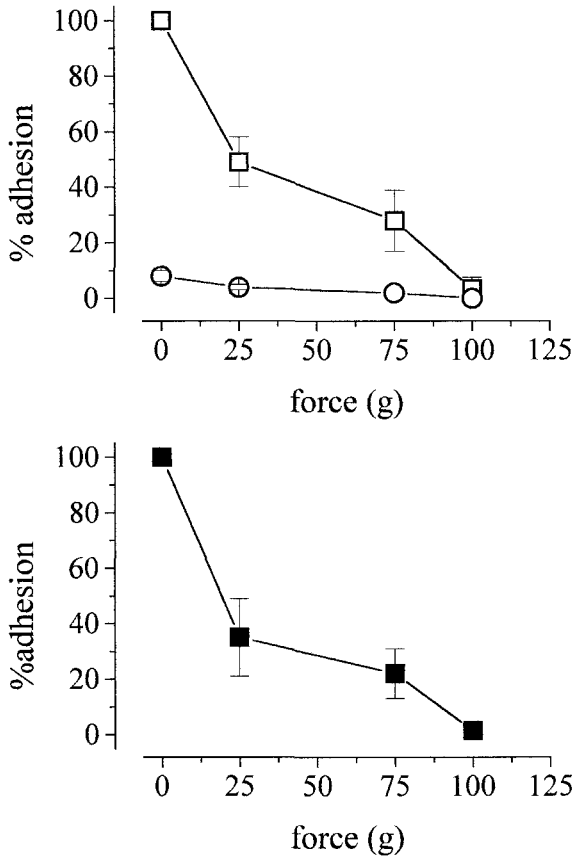

Fig. 2. Laminin-α1-mediated adhesion of control and *mdx* mouse myotubes. Upper panel: Experiments using normal myotubes (□) and an integrin subunit β_1 antibody (○, see Section 3.1). Lower panel: Experiments using *mdx* myotubes. Each point is the average of five experiments; the average number of cells/petri was 472 ± 30.

assess the adhesion of the *mdx* myotubes, as shown in Fig. 2. The experiments were performed using both normal (Fig. 2, upper panel) and *mdx* (Fig. 2, lower panel) mouse myotubes. It is clear that the *mdx* and control myotubes adhere in a similar manner thus suggesting that the major mechanisms sustaining the adhesion to laminin-α1 may be different from the supposed α-dystroglycan-mediated link. The use of an antibody raised against the β_1-subunit [19] of mouse integrin severely impaired any type of adhesion on laminin-α1 (Fig. 2, upper, ○).

3.2. *'mdx' but not normal myotubes are unable to adhere to laminin-α2*

The results of the similar experiments performed using laminin-α2 are shown in Fig. 3 (left). Under control conditions the degree of adhesion was remarkably high at a laminin-α2 concentration of 5 μg/ml, but the experiment involving *mdx* myotubes showed very poor adhesion: at 75 g, the normal myotubes showed 95% adhesion as against the 5% adhesion of *mdx* myotubes. On the whole, these results suggest that laminin-α2 selectively reveals a striking difference in the level of the adhesion force used by normal and *mdx* myotubes to contact ECM.

3.3. *DMD myotubes are also unable to adhere to laminin-α2 whereas normal myotubes are almost undetachable.*

We used human normal and DMD myotubes to repeat the mouse experiments described above. As shown in Fig. 3 (right), the results were qualitatively very similar. Given that formed human myotubes are particularly large and delicate, we had to culture myoblasts directly on laminin-α2 substrates; this led to such strong adhesion that it was almost impossible to detach the myotubes even at very high forces. Although the myotubes in the DMD cultures were somewhat smaller and thus less inclined to be detached (see Section 2), we found the same interesting difference as that shown for mouse: the DMD cells detached at a force that was several times less than that necessary to detach normal cells. Control experiments (not shown) with human myotubes on substrata coated with laminin-α1 showed a 50% adhesion at 600 g for normal myotubes but only at 800 g for DMD myotubes, a

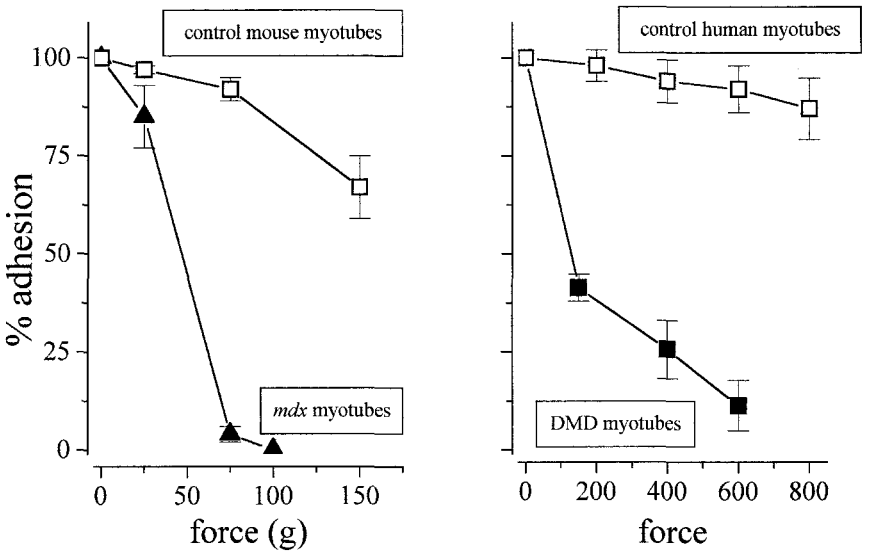

Fig. 3. Adhesion of normal and *mdx* mouse myotubes (left) and human DMD myotubes (right) to laminin-α2 substrata. Left panel: Notice that the *mdx* myotubes adhered to the laminin-α2 substratum much less strongly than the normal myotubes. Each point is the mean of four experiments; the average number of cells/petri was 610 ± 24. Right panel: Note that DMD myotubes can be detached at forces at which normal myotubes are virtually undetectable. Each point is the mean of three experiments; the average number of cells/petri was 900 ± 42.

result that is in keeping with our previous observations, suggesting that laminin-α1 is incapable of recognizing the difference between normal and DMD muscle cells.

On the whole, the results on human myotubes were in complete agreement with those observed in mouse, and suggest that the inability to adhere strongly to laminin-α2 is peculiar to Duchenne and *mdx* diseases, in which myocells are known to lack dystrophin and α-dystroglycan.

4. Discussion

The results reported in the present paper demonstrate that dystrophic myotubes (from *mdx* mice and DMD patients), which are known to lack α-dystroglycan, are incapable of correctly contacting laminin-α2 although they do correctly adhere to normal laminin-α1.

It has recently been shown that laminin-α2 and not laminin-α1 are expressed in 14–17-day-old mouse embryos at the level of the muscle fibre basement membranes. Moreover, myoblasts can spread much faster on laminin-α2 than -α1, thus suggesting that the functional roles of the two forms may be quite different [20]. Furthermore, a number of studies [21–23] suggest the presence of integrins $\alpha_7\beta_1$ in myotubes although no conclusions can yet be drawn as to whether the same integrin also mediates binding to laminin-α2 [24]. We found that laminin-α1 perfectly supports the adhesion of *mdx* myotubes lacking α-dystroglycan: the obvious conclusion is that this type of adhesion is sustained by other receptors (putatively $\alpha_7\beta_1$) and that the possible disorganisation of the *mdx* membrane and cytoskeleton does not affect these receptors. It has previously been shown [25] that *mdx* are more fragile than normal myotubes, but our data indirectly indicate that this fragility is restricted to the level of the cytoskeleton, and does not involve the sarcolemma/ECM interface, which functions normally when laminin is present. It is becoming increasingly clear that muscular dystrophy may be caused by mutations in a number of the proteins involved in the stabilisation of muscle cell membrane, including the attachment of cell to the ECM. In this respect, some human (congenital muscular dystrophy, CMD and Fukuyama-type congenital muscular dystrophy, FCMD) and mouse (*dy*) muscular dystrophies have been shown: (i) to depend on the deficiency or incorrect functioning of laminin-α2 which binds α-dystroglycan [26,27] and (ii) to cause muscle weakness similar to Duchenne and Becker dystrophies. On the whole, our results suggest that the DMD defect, (which is specular to that of CMD) may have the direct consequence of impairing the assembly of muscle fibers in a complicated and still unclear manner.

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