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Original articles

An attempt of gene therapy in Duchenne muscular dystrophy : overexpression of utrophin in transgenic mdx mice

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Abstract

Dystrophin, its functions and the consequences of its absence are briefly reviewed. The animal model of Duchenne myopathy, the mdx mouse, was used to over-express utrophin by transgenesis technology. A battery of functional tests, including mechanical responses (force development and resistance to imposed stretch), intracellular calcium homeostasis and metabolic reaction to muscle activity were applied to check the functional recovery obtained by over-expression of utrophin. For most parameters tested, recovery amounted to 80%, demonstrating that utrophin can very efficiently act as a surrogate for dystrophin.

Key words : Duchenne dystrophy ; dystrophin ; gene therapy ; utrophin ; mdx mouse.

Introduction

THE DISEASE

Duchenne muscular dystrophy (DMD) is a greatly disabling disease in man. It is usually detected around the age of 2-3 years, on the basis of difficulties in walking. These difficulties worsen and by the age of 10-12 years the patient needs a wheelchair. The disability is due to the progressive degeneration of skeletal muscles with replacement by fat deposits and proliferative fibrosis. Muscle wasting affects trunk and limb muscles and important deformations of the spine later develop which impair ventilation. Later, the patient is obliged to lie in bed and death occurs around the age of 20, by respiratory and cardiac failure. To date, the disease cannot be cured.

Duchenne myopathy affects males only. It is a sex-linked genetic disease, affecting 1/3500 male births. Since 1987, it is known that the disease results from mutations affecting an enormous gene of about 2 millions base pairs, located on the short arm of chromosome X in the p21 region. The disease is transmitted by heterozygous, non-affected women. Unfortunately, about 1/3 of the cases are *de novo* mutations, unexpected and thus undetectable by genetic analysis of the family tree.

THE GENE AND ITS PRODUCT

The affected gene codes for a 427 kDa protein, resulting from a RNA messenger of 14.5 kb, resulting from 79 exons. The protein, coined "dystrophin", is normally localized under the plasma membranes of striated, cardiac and smooth muscles fibres (Hoffman *et al.*, 1987). Depending on the type of mutation, transcription of dystrophin stops prematurely and the product is degraded. This situation is found in severe Duchenne phenotypes. If the mutation did not affect the reading frame, often an incomplete protein, more or less shortened in its central region, but with normal N and C terminals, may appear. In these cases, the phenotype is much less severe, even mild, and is known by clinicians as Becker myopathy (Hoffman *et al.*, 1988).

Mutations in the dystrophin gene have been discovered in several animal species. The dmx dog (Golden Retriever) presents a very severe phenotype (which makes its reproduction rather difficult) and the mdx mouse (mutant of the wild type C57/BL10), wherein, after a severe crisis of muscle degeneration around the age of 4 weeks, the evolution of the disease is rather mild, allowing the establishment of a mutant line. In both cases, dystrophin is totally absent from muscle fibres. The mdx mouse is the most used animal model for experimental studies on Duchenne muscular dystrophy.

PHYSIOLOGICAL ROLE OF DYSTROPHIN

This role is still largely unknown. Present views come from the observations of abnormalities detected in dystrophin-deficient muscles. The following is an abbreviated version of comprehensive reviews (Gillis, 1996 ; Gillis, 1999) recently published by the author, where essential references can be found.

Structural role. Studies of structural interactions

between proteins have revealed that dystrophin reacts through its N-terminal with filamentous actin of the cytoskeleton underneath the plasma membrane (this is not the myofilament actin). Through its C-terminal, dystrophin interacts with a complex of membrane-associated glycoproteins (so called *dystrophin-associated proteins, DAG*), composed of several subunits including ßdystroglycan with which dystrophin directly interacts. On the extracellular side, this complex interacts with laminin (also called merosin) and the latter with collagen IV. These protein-protein interactions make a continuous mechanical link between the sub-membranous cytoskeleton and the extracellular matrix and, in this continuity, dystrophin plays an essential role. This membrane-linked protein architecture is illustrated at the Fig. 1 (see (Matsumura and Campbell, 1994) and (Winder, 1997) for reviews).

Indeed, when dystrophin is absent, the DAG, though normally expressed, are no longer correctly addressed to the membrane and are rapidly degraded. These results led to the hypothesis that the absence of dystrophin "fragilizes" the muscle fibres that are submitted to the high mechanical strain developed during contraction.

It was shown in our laboratory that the dystrophin-deficient muscles (from mdx mice) are particularly susceptible to contractions with forced lengthening (Moens *et al.*, 1993), a type of contraction known as "eccentric contraction" in sport physiology. When a fully tetanized muscle is submitted to forced elongation, the isometric tension developed during the next contraction is reduced. This irreversible drop of force results from membrane microlesions which can be detected by the intracellular penetration of extracellular dyes (e.g., Evans blue or Orange procion). These results confirmed the hypothesis of membrane fragility that can be detected in precise experimental conditions. It is worth to note that eccentric contractions are not laboratory oddities : they occur during integrated muscle movements as walking or going down a stair, every time when the movement of a body segment must be slowed down.

Role in the intracellular calcium homeostasis. The implication of dystrophin in this fundamental property is very much controversial. It is based on the following observations : (1) the total calcium content of dystrophin-deficient muscles is about doubled, but it is not known if this is a cause or a consequence of the dystrophic process. (2) Myotubes (incompletely differentiated fibres) present leak channels for calcium which show abnormally long opening times. The persistence of these channels in the adult fibre is controversial. (3) An American group reported that the cytosolic concentration of calcium, $[Ca^{2+}]_i$, was chronically elevated in dystrophin-deficient fibres (Turner *et al.*, 1988 ; Turner *et al.*, 1991). We showed that this was not the case (Gailly *et al.*, 1993) and our observation has been confirmed by three other laboratories (see review in Gillis, 1999). Myotubes from mdx mice submitted to the combination of an

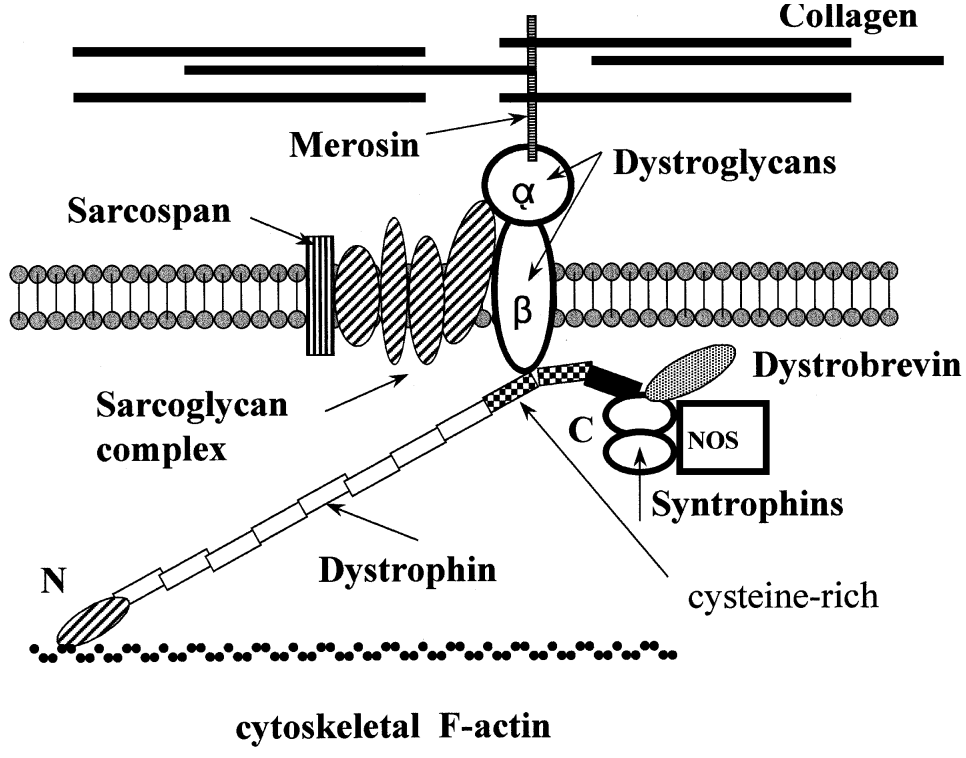

Fig. 1. — Schematic view of the protein-protein interactions involving dystrophin, the sarcodystroglycan complex inserted into the sarcolemma and the connection with the extracellular matrix. Various proteins specifically bound to dystrophin are also shown, together with the interaction with the subsarcolemmal actin cytoskeleton. NOS = nitric oxide synthase.

hypo-osmotic + hypercalcic (x 10) medium, show abnormal elevation of $[Ca^{2+}]_i$ (Imbert *et al.*, 1996). (5) Elevation of extracellular calcium, increases the turnover of proteins in mdx muscles (Turner *et al.*, 1988).

These observations do not allow a coherent conclusion but indicate that in certain experimental conditions it is possible to induce a deficit in the intracellular calcium homeostasis. If this occurred in vivo, this might activate aspecific Ca^{2+}-dependent proteases and lead to fibre necrosis (for a detailed discussion see Gillis, 1999).

A gene therapy strategy

The mdx mouse, deficient in dystrophin, was the first animal model submitted to the trials for gene therapy. I here report a summary of a collaborative work involving a team of geneticists from Oxford University led by Prof.K.Davies and our physiology group at the Catholic University of Louvain (UCL) (Deconinck *et al.*, 1997 ; Goudemant *et al.*, 1998 ; Tinsley *et al.*, 1998)

Gene therapy by expression of utrophin

Cross sections of mdx muscles treated with anti-dystrophin polyclonal antibodies revealed "positive spots" that turned out to be located at the neuro-muscular junctions and the myotendinous junctions (Fardeau *et al.*, 1990). This observation led to the discovery of a new protein, very close to dystrophin (80% homology,(nsley *et al.*, 1992) but coded on an autosome (chromosome 6, in man ; 15 in the mouse), thus unaffected in mdx mice and Duchenne patients. It was coined "utrophin" as it is expressed in many cell types (including muscle fibres) during development. In adult muscles, however, its expression, for unknown reasons, is limited to neuro-muscular and myotendinous junctions. When specific antibodies were available, it was found that in dystrophin-deficient muscles, utrophin was slightly overexpressed and could be detected along the whole periphery of the fibre membrane, as if the absence of dystrophin "de-repressed" the expression of utrophin (Mizumo *et al.*, 1993). Given the high homology between the two proteins, the hypothesis was put forward that, if expressed in large quantities, utrophin possibly could be a functional surrogate for dystrophin (Tinsley and Davies, 1993).

Using the transgenesis methodology, J.Tinsley and K.Davies in Oxford, have obtained transgenic mdx mice where large quantities of a truncated form of utrophin was expressed in skeletal muscles (under the influence of the strong promoter of skeletal muscle actin). This utrophin was deleted in its central, rod-like part, but both N and C terminals were preserved. Skeletal muscles from these adult transgenic mice show little signs of dystrophy and recover the correct localization of the DAG (Tinsley, 1996). This last point indicated that, structurally, truncated utrophin was able to replace dystrophin.

The evaluation of the success of the gene therapy : the functional recovery

A decisive criterion for judging of the success of a gene therapy protocol is the functional recovery. It is not sufficient to demonstrate that the protocol succeeded in obtaining the expression of the protein to be tested, it is essential that this protein is functionally active.

We designed a battery of functional tests with a high level of discrimination between dystrophin-deficient and normal muscles. These tests, when applied to muscles from mice submitted to gene therapy assays, provide objective and quantitative means for judging the success of the therapy.

Tests of the contractile properties. This is essential for cells the main function of which is to provide mechanical energy. The isometric force of isolated muscles from mdx, treated or control mice was first systematically measured, as it is diminished in mdx muscles. Then, muscles were submitted to series of contractions with forced lengthenings (called eccentric contractions), a condition for which, mdx muscles show great sensitivity reflected by a progressive loss of force. It is a very exacting and discriminating test that reveals micro-lesions occurring during eccentric contractions. These damages have also been documented by the penetration of extracellular dyes.

The overall force of the muscular system of the living animal was also monitored by the force developed during an "escape test" (pinching of the tail connected to a force transducer). In spite of its simplicity, this non-invasive test allows to quantify the recovery obtained.

Tests of the calcium homeostasis. It was studied first by measuring the total calcium content, which is doubled in mdx muscles. We also designed a test to challenge the capacity of the fibre to maintain a low $[Ca^{2+}]_i$ while bathing in an hypo-osmotic + hypercalcic medium. The evolution of $[Ca^{2+}]_i$ during the test shows a deficiency of the mdx fibre to maintain a low $[Ca^{2+}]_i$.

Tests of the metabolic regulations. During a series of repeated contractions (*in vivo* fatigue test in anaesthetised animals), the use of high-energy phosphate compounds (ATP, PCr) was monitored by nuclear magnetic resonance of ^{31}P, using a specialised miniaturised antenna. The evolution of the intracellular pH was also monitored as it was known that fatigue led to deeper acidosis and slower recovery in mdx muscles.

Besides these functional tests, histological examination was performed to determine the per-

centage of centrally nucleated fibres (a classical marker of cycles of degeneration-regeneration) together with measurements of serum creatine kinase activity.

Results

The work was based on three lines of transgenic mice showing a strong expression of truncated utrophin (trc-utrophin) on immunohistological frozen sections (called hereafter Utr++ lines) and on one line where the expression was about three times lower (Utr+ line).

Here below are summarised the characteristics we observed in muscles of these transgenic mice.

Histological and systemic parameters :

(1) Trc-utrophin was expressed along the whole periphery of the fibres in all transgenic lines. In Utr++ lines, the percentage of centrally nucleated fibres was much reduced (this was particularly impressive in diaphragm, the most affected muscle in mdx mice). This effect was less pronounced in Utr+ lines.

(2) In all lines (Utr++ and Utr+), serum creatine kinase level is returned to normal values.

(3) The pseudo-hypertrophy, so characteristic of the mdx phenotype is reduced.

Tests of the contractile response :

(4) Important recovery of the isometric force.

(5) Important increase of the resistance to eccentric contractions.

(6) Important increase of the muscle force *in vivo*, tested by the escape test.

The recovery of the contractile properties was larger in Utr++ lines than in the Utr+ line.

Tests of the calcium homeostasis :

(7) Reduction of the total calcium content, more pronounced in Utr++ muscles.

(8) Normalisation of the $[Ca^{2+}]_i$ response to the challenge combining hypo-osmotic + hypercalcic medium.

Tests of the response to metabolic perturbations. During repeated stimulation of the gastrocnemius muscle *in vivo*, we observed, in these same Utr++ transgenic mice, a significant reduction of the intracellular acidosis compared with mdx muscles. During the post-contractile period, pH of Utr++ muscle returned to normal values with a normal time course, while acidosis remained present in mdx muscles.

Thus, expression of utrophin, even in the truncated form, radically changed the muscle phenotype. This can best be expressed as the "*recovery score*", where 100% means complete recovery of the normal properties and 0% no improvement over the mdx situation.

The parameters which are the most affected in mdx muscles present the large recovery score around 70-85%. If one assumes that dystrophin and utrophin are functionally interchangeable molecules on a 1/1 basis, then it can be estimated from various published data and from ours that trc-utrophin expression in the Utr++ lines is equivalent to about 40% of dystrophin expression in normal muscles. This level is much higher than the one that occurs spontaneously in the muscles of mdx mice or DMD patients. However, it is possible that even this low spontaneous expression plays some compensating role which delays the fatal outcome of the disease as suggested by the following.

Confirmation a contrario *of the compensatory role of utrophin.* We also studied mice presenting the combined deficiency of dystrophin and utrophin (mdx mice where the utrophin gene was deactivated, obtained by our Oxford colleagues J. Rafael and K. Davies). Doubly deficient animals hardly survive over two months. Their isolated muscles showed a great weakness of the force development and a dramatic collapse of the resistance to eccentric contractions (Deconinck *et al.*, 1998).

Obtaining transgenic mdx mice where the complete utrophin molecule was overexpressed later complemented these studies. In this case, only the mechanical parameters were examined. Complete recovery of force development and resistance to stretch were obtained (Tinsley *et al*., 1998).

Short conclusions and perspectives

Gene therapy of Duchenne myopathy is no longer a utopia. Our experimental work demonstrated that it is possible to repair, in dystrophin-deficient fibres, the functional characteristics of the normal fibre and thus to avoid deficiencies which cause the degeneration of the muscle fibres. When utrophin expression is amplified, even in its truncated form, so that the protein is present along the whole periphery of the fibre, the recovery of numerous cellular functions, most importantly contractile, reaches about 80%. If extrapolated to the human DMD patient, such a recovery would transform the 100% fatal Duchenne disease into a very mild Becker myopathy. It is worth to note that this approach is not prone to induce unwanted immunological reactions as utrophin is a normal component of dystrophin-deficient muscles.

Passing from the animal model to the DMD human patient in order to obtain a cure of the Duchenne disease will require that the therapy can affect the whole of the skeletal muscles. This will require the discovery of factors, natural or synthetic, able to switch on strongly and permanently

the utrophin gene. To attain this goal, challenges and hurdles to be overcome are still formidable but large scientific and financial investments are really worthwhile, since we demonstrated that "utrophin therapy" is efficient to obtain a very important functional correction.

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