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Cell surface and gene expression regulation molecules in dystrophinopathy: *mdx* vs. Duchenne

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ABSTRACT

Duchenne muscular dystrophy (DMD) is secondary to loss-of-function mutations in the dystrophin gene. The causes underlying the progression of DMD, differential muscle involvement, and the discrepancies in phenotypes among species with the same genetic defect are not understood. The *mdx* mouse, an animal model with dystrophin mutation, has a milder phenotype. This article reviews the available information on expression of signaling-related molecules in DMD and *mdx*. Extracellular matrix proteoglycans, growth factors, integrins, caveolin-3, and neuronal nitric oxide synthase expression do not show significant differences. Calcineurin is inconsistently activated in *mdx*, which is associated with lack of cardiomyopathy, compared to the permanent calcineurin activation in *mdx*/utrophin null mice that have a DMD-like cardiomyopathy. Levels of focal adhesion kinase (FAK) and extracellular regulated kinases (ERKs) differ among *mdx* and DMD. Further work is needed to identify the point of discrepancy in these signaling molecules' pathways in dystrophynopathies.

Key terms: dystrophinopathy, signaling molecules, Duchenne muscular dystrophy.

Duchenne Muscular Dystrophy (DMD) is a muscle degenerative disease that is invariably fatal in humans. The primary cause of DMD is a mutation in the dystrophin gene leading to the absence of the corresponding RNA transcript and protein. It is believed that the absence of dystrophin leads to disruption of the dystrophin-associated protein complex, producing sarcolemmal instability that could render muscle fibers susceptible to necrosis (Matsumura et al., 1994). Although the histological pathology of dystrophic tissue has been well documented, little is known about the cascade of molecular events triggered by the absence of dystrophin that lead to muscle degeneration. Impairment of calcium homeostasis has been postulated as a key process but remains controversial (Gailly, 2002). The study of the specific sources of calcium signals involved in regulation of gene expression in skeletal muscle has been addressed only recently (Carrasco et al, 2004) and is beyond the scope of this article. There is an animal model of lack of dystrophin, the *mdx* mouse. The mutants show mild clinical symptoms and are viable and fertile (Bulfield et al., 1984), in contrast to the fatal human disease. Different functional results in both species with the same genetic defect suggest that there is a distinct cellular response in each case. This article reviews what is known about differences in signal transduction and related molecules' expression in both model and our contribution to the subject. This text is organized in three segments, progressing from the outside of the muscle cell towards the inside: muscle surface and extracellular space, sarcolemma, and sarcoplasma.

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EXTRACELLULAR SPACE

It is known that in dystrophinopathies there is fibrosis in the interstitial space, that is characterized by the accumulation of extracellular matrix (ECM) material (Rampoldi et al., 1986). The ECM acts regulating growth factors and cytokines activation status and turnover: several of its molecules have signaling functions, therefore, it likely has an important role in muscle fibrosis and phenotype regulation. The ECM constituents that may participate regulating signaling are adhesion molecules, proteoglycans, and growth factors.

Proteoglycans

The total synthesis of proteoglycans, an ECM main constituent, is increased in *mdx* mice (Cáceres et al., 2000). Muscle satellite cells from dystrophic mice have elevated levels of heparan sulphate proteoglycan receptors for fibroblast growth factor and synthesize ten times more sulfated glicosaminoglycans in culture than normal cells (Crisona et al., 1998). Biglycan, a small leucine-rich proteoglycan, shows increased expression in muscle from the dystrophic *mdx* mouse (Bowe et al., 2000). We found that heparan sulfate proteoglycans are increased in muscle biopsies of DMD patients (Álvarez et al., 2002). Through SDS-PAGE analyses followed by specific identification of heparitinase-digested proteins with anti-Delta-heparan sulfate specific monoclonal antibodies, we observed an increase of three forms of heparan sulfate proteoglycans, corresponding to perlecan, syndecan-3, and glypican-1. Immunohistochemistry analyses indicated a differential localization for these proteoglycans: glypican-1 and perlecan were found mainly associated to ECM structures, while syndecan-3 was associated to muscle fibers (Álvarez et al., 2002). We also found that chondroitin/dermatan sulfate proteoglycans, decorine, and biglican are increased in DMD patients (Fadic et al., in preparation). The only information available about decorin and biglycan levels in DMD comes from gene expression profiles of skeletal muscle biopsies that demonstrate increases in their amount (Haslett et al., 2002). In both DMD patients and *mdx* mice, there is a general increase in the amount of proteoglycans. Although further work is needed, it does not appear that there will be major differences between both species.

Growth factors

Three growth factors are related to muscle dystrophy: fibroblastic growth factor (FGF), transforming growth factor beta-1 (TGF-β1) and its related molecule myostatin, and insulin-like growth factor-I (IGF-1). A heightened sensitivity of *mdx* satellite cells to FGF was described (DiMario and Strohman, 1988). FGF levels in the *mdx* extracellular matrix are higher than those in controls (Anderson et al., 1991; DiMario et al., 1989). FGF is co-expressed with myogenin in adjacent sections of focal muscle degeneration and repair in *mdx* (Garrett and Anderson, 1995). Pre-treating myoblasts with FGF increases the percentage of fibers expressing dystrophin in host *mdx* muscle (Kinoshita et al., 1996). FGF-deficient myoblasts have reduced motility that may contribute to the dystrophic changes in FGF2/FGF6/*mdx* triple-mutant mice (Neuhaus et al., 2003). Not much is known about FGF in DMD, but an elevated FGF level in the serum of patients was reported (D'Amore et al., 1994). In DMD, high TGF-β 1 immunoreactivity was expressed on muscle fibers and extracellular space. In other myopathies with endomysial fibrosis, however, TGF-beta 1 was seldom observed (Yamazaki et al., 1994). The TGF-β1 mRNA levels were greater in DMD patients than controls (Bernasconi et al., 1995). A similar result was reported in *mdx* diaphragm (Gosselin et al., 2004). The failure of myoregeneration in DMD has been related to TGF-β1 released from necrotic muscle fibers (Melone et al., 1999). Plasma TGF-β1 level was significantly elevated in patients with DMD and congenital muscular dystrophy (CMD) but not in those with Becker muscular dystrophy (Ishitobi et al., 2000). Using expression microarrays analysis, overexpression of TGF-β1 was found in DMD (Haslett et al., 2002). Myostatin, a TGF- β family member, is a

negative regulator of muscle growth. It was found to be down-regulated in dystrophic muscle (Zhu et al., 2000). Blockade of endogenous myostatin by using intraperitoneal injections of blocking antibodies resulted in an improvement of the phenotype of the *mdx* mouse (Bogdanovich et al., 2002); a similar result was obtained with a myostatin-null *mdx* mouse (Wagner et al., 2002). IGF-1 is known to promote proliferation and differentiation of muscle cells during growth and regeneration. At 8-10 weeks of age, significantly greater amounts of IGF-1 were found in plasma and hind limb muscles of *mdx* mice with respect to controls. Such a difference was only just detectable and no longer statistically significant at 5 months of age. No differences were found in hepatic IGF-1 levels at either age (De Luca et al., 1999). Interestingly, IGF-1 expression significantly reduced the amount of fibrosis and myonecrosis in diaphragms from *mdx* mice (Barton et al., 2002). Administration of IGF-1 improved contractile function in mdx (Gregorevic et al., 2002). An increased expression of IGF-binding protein-5 in DMD fibroblasts *in vitro* was described (Melone et al., 2000). Expression microarrays were used to compare individual gene expression profiles of skeletal muscle biopsies from DMD patients and control patients. Bakay reported transcriptional up-regulation of both IGF-1 and IGF-2 in dystrophic muscle, however, the possible beneficial effects of the growth factors appear to be offset by transcriptional up-regulation of inhibitory IGF-binding proteins and regulators (Bakay et al., 2002). Additionally, an increase in expression of two IGF regulators: IGF-binding protein 4 (IGFBP4) and serine protease 11 was reported, without evidence of over-expression of IGFs (Haslett et al., 2002). No notable differences have been described in growth factors levels or functions of DMD and *mdx*.

SARCOLEMMA

The α7β1 integrin is the primary laminin receptor on myofibers. It has distinct functions during muscle development and contributes to muscle structural integrity. Immunofluorescence demonstrates an increase in α7β1 in patients with DMD and in *mdx* mice. Analysis of RNA from both DMD patients and *mdx* indicates that the increase in the α7β1 integrin is regulated at the level of α7 gene transcription (Hodges et al., 1997). Enhanced expression of the α7 chain in transgenic *mdx/utr*(-/-) mice, which lack both dystrophin and utrophin, alleviates many of the symptoms of dystrophy and compensates for the absence of the dystrophin- and utrophin-mediated linkage systems (Burkin et al., 2001). Nitric oxide (NO) is synthesized in skeletal muscle by neuronal-type NO synthase (nNOS), which is localized to sarcolemma of fast-twitch fibers associated to dystrophin. Synthesis of NO in active muscle opposes contractile force. Both *mdx* mice and humans with DMD show a selective loss of nNOS protein and catalytic activity from muscle membranes (Brenman et al., 1995; Chang et al., 1996). nNOS is relocated to the interior of muscle cells, where it continues to produce NO, but this does not contribute to dystrophy pathogenesis, as shown in nNOS-dystrophin null mice (Crosbie et al., 1998). Caveolae are vesicular invaginations of the plasma membrane and function as "message centers" for regulating signal transduction events. Caveolin-3, a muscle-specific caveolin-related protein, is the principal structural protein of caveolar membrane domains in skeletal muscle. The density of caveolae is increased in DMD and *mdx* mouse (Repetto et al., 1999; Vaghy et al., 1998). Caveolin-3-overexpressing transgenic mice has a dystrophic phenotype (Galbiati et al., 2000). There is, then, no major difference between DMD and *mdx* with regard to integrins, caveolin-3, and nNOS expression.

SARCOPLASMA

Ca2+/calmodulin-activated phosphatase calcineurin is ubiquitously expressed. Upon activation by Ca2+, calcineurin dephosphorylates nuclear factor of activated T cell (NFAT) transcription

factors, leading to their nuclear translocation. Dilated cardiomyopathy is a common complication of DMD; in contrast, the *mdx* mouse shows mildly dystrophic changes in the heart. The utrophin-dystrophin knockout (dko) mouse shows severe dystrophic changes in cardiac muscle that more closely resembles DMD cardiomyopathy than *mdx* mouse. Interestingly, calcineurin and SAPK/p38-MAPK signaling pathways were constantly activated in dko hearts, but the activation varied in *mdx* hearts (Nakamura et al., 2001). Mice expressing enhanced muscle calcineurin activity (CnA*) display elevated levels of utrophin around their sarcolemma. Crossing CnA* mice with *mdx* mice attenuates the dystrophic pathology (Chakkalakal et al., 2004). Deflazacort attenuates loss of dystrophic myofiber integrity in DMD muscle cells by up-regulating the activity of calcineurin (St-Pierre et al., 2004).

There is very little information about extracellular regulated kinases (ERKs), a common endpoint for several signaling pathways, in both DMD and mdx. Levels of ERKs as well as phosphorylation states were elevated in the diaphragm and limb muscle of mdx mice compared with age-matched control muscles (Lang et al., 2004). We found increased levels of total ERKs in both *mdx* and DMD. However, the phosphorylated ERK form is only increased in DMD (Muñoz, Mezzano, Brandan and Fadic, submitted to PNAS USA). Focal adhesion kinase (FAK) is an important protein tyrosine kinase that mediates several integrin signaling pathways. There is no published information about FAK in dystrophinopathies. It is decreased in the *dy* mouse, an animal model for the merosin-negative congenital muscular dystrophy (Sakuma et al., 2004). On the contrary, an increased level of activated FAK was found in muscle of α7 integrin-deficient mice (Saher and Hildt, 1999). We found no changes in FAK levels in *mdx*, but a reduced level in DMD (Muñoz, Mezzano, Brandan and Fadic, submitted to PNAS USA). Additional work is needed to identify the reasons for this divergence. Understanding the different cellular responses in both species to a similar injury may allow us to understand the pathogenesis of the muscle cell damage in this currently lethal condition.

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