In vitro dissection of the pathogenic mechanisms of muscle fibrosis in Duchenne muscle dystrophy

PhD thesis of Zanotti Simona

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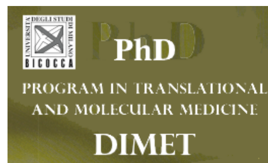

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Chapter 1

1.1 Muscular dystrophy

Introduction

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive muscle-wasting disease (OMIM#310200) affecting all world populations equally, with an incidence of approximately 1 in 3500 live male births (Brown SC and Lucy JA 1993).

DMD was named in recognition of Dr. G. Duchenne de Boulogne from France, who was the first to attribute the signs and symptoms to a distinct familial disease entity about 150 years ago, originally describing the condition as *paralysie pseudo-hypertrophique* ("pseudo-hypertrophic muscular paralysis").

Becker muscular dystrophy (BMD), a milder form of the disease, has a later onset and a much longer survival. DMD and BMD are both caused by mutations in the *DMD* gene that encode a 427-kDa cytoskeletal protein called dystrophin. The vast majority of DMD mutations result in the complete absence of dystrophin, whereas the presence of lower levels of protein or of a truncated molecule is seen in BMD patients.

A. Clinical progression of DMD and BMD muscular dystrophies

Typically, DMD patients are clinically normal at birth although serum levels of the muscle isoform of creatine kinase are elevated as a consequence of muscle fiber degeneration. Initial physical signs such as

muscle weakness are not generally observed until the child reaches 2-5 years of age. Signs are typified by a waddling gait and difficulties in running and climbing stairs. Subsequent onset of pseudohypertophy of the calf muscles, proximal limb muscle weakness, a positive Gowers' sign (Gowers W, 1879) or the child's use of arms to climb from a lying to standing position clinically suggest DMD.

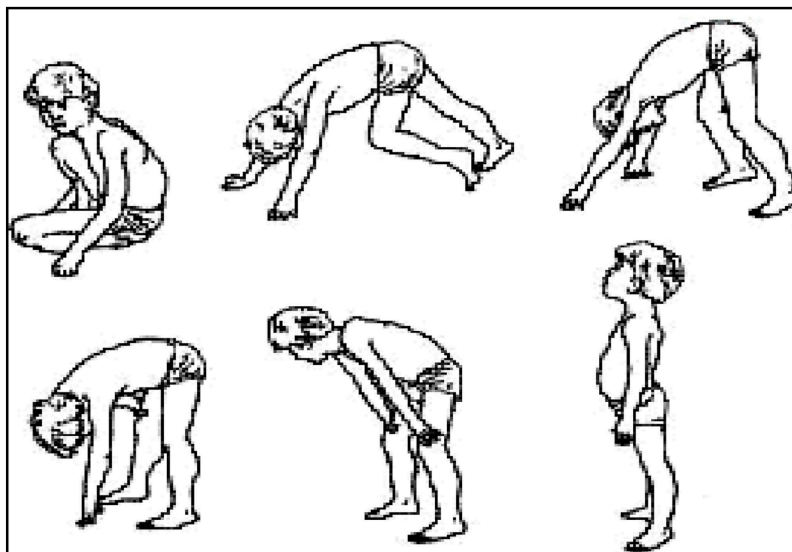


Fig. 1. Gowers' sign: child's use of arms to climb from a lying to a standing position.

Progressive muscle wasting continues throughout life, initially affecting proximal muscles, with a decrease in lower limb muscle strength resulting in loss of ambulation by about 12 years of age. In the latter stages almost all skeletal muscles are severely involved, which in turn leads to additional problems such as joint contractures and progressive kyphoscoliosis.

In addition to musculoskeletal deficit, some DMD patients have central nervous system disorders such as cerebral atrophy (Yoshioka M et al, 1980) and abnormal dendrite branching (Jagadha V and Becker LE, 1988), causing mild mental retardation and behavioural problems in 20-30% of DMD patients (Blake DJ and Kroger S, 2000). This also contributes to a diminished quality of life. As the disease progresses, ventilatory assistance is required, and, ultimately, premature death occurs in DMD patients in their early twenties from cardiac dysfunction and respiratory failure. Notably, recent improvements in medical therapy and support in the Western world are pushing this interval to early thirties and beyond.

In individuals affected by BMD, the clinical course is similar to that of DMD, although the onset of symptoms and the rate of progression are delayed. More than 90% of patients are still alive in their twenties, with some patients remaining mobile until old age.

Historically, the varied clinical course and milder phenotype let to a reluctance in classifying these patients with DMD, until an elegant hypothesis concerning its allelic nature of disorder was proposed by Becker and Kiener (Becker E and Keiner F, 1955). Both BMD and DMD patients can present with varying degrees of cognitive impairment, indicating that brain function is abnormal in these disorders.

DMD AND DYSTROPHIN GENE PRODUCTS

Twenty years ago the genetic defect underlying DMD was mapped to chromosome Xp21 in humans (Monaco AP et al, 1988). This gene is the largest identified to date, which could account for its relatively high frequency of mutation and incidence of DMD (Coffey AJ et al, 1992).

The human DMD locus spans approximately 2.5 Mb of genomic sequence, the *DMD* gene consists of 79 exons (Koenig M et al, 1987).

The *DMD* gene can accommodate production of several dystrophin isoforms through alternative promoter usage and splicing of pre-mRNA. Three independently regulated promoters control expression of the full-length dystrophin transcript. The brain (B), muscle (M), and the Purkinje (P) promoters consist of unique first exons spliced to common set of 78 exons (Fig. 2). The names of these promoters reflect the major site of dystrophin expression. The B promoter drives expression primarily in cortical neurons and the hippocampus of brain (Barnea E et al, 1990), while the P promoter is expressed in the cerebellar Purkinje cells and also skeletal muscle (Gorecki DC et al, 1992; Holder E et al, 1996). The M promoter results in high levels of expression in skeletal muscles and cardiomyocytes and also at low levels in some glial cells in the brain (Barnea E et al, 1990).

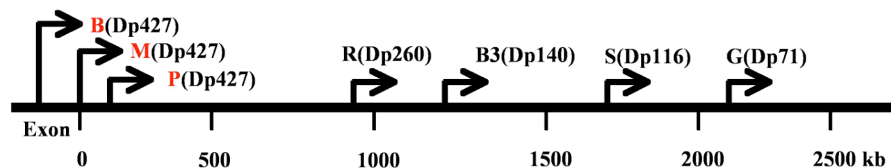


Fig. 2. The organization of human *DMD* gene. The "full-length" dystrophin transcripts are transcribed from promoters in the 5'-end of the gene. Each mRNA encodes a 427-kda protein that only differs in the NH_2-terminal sequence. The three products are designed Dp427 (B, brain), Dp427 (M, muscle) and DP427 (P, Purknje cells) to reflect their tissue-specific expression pattern.

B. Dystrophin isoforms and splice variants

The *DMD* gene also has at least four internal promoters that give rise to shorter dystrophin transcripts that encode truncated COOH-terminal isoforms. These internal promoters can be referred to as retinal (R), brain-3 (B3), Schwann cell (S) and general (G). Each of these promoters utilizes a unique first exon that splices in two exons 30, 45, 56 and 63 respectively, to generate protein products of 260 kDa (Dp260), 140 kDa (Dp140), 116 kDa (Dp116) and 71 kDa (Dp71) (Fig. 3). Dp71 is detected in most nonmuscle tissues including brain, kidney, liver, and lung (Blake DJ et al 1992; Howard PL et al, 1999; Howard PL et al, 1998; Hugnot JP et al, 1992), while the remaining short isoforms are primarly expressed in the central and peripheral nervous system (Lidov HG et al, 1995).

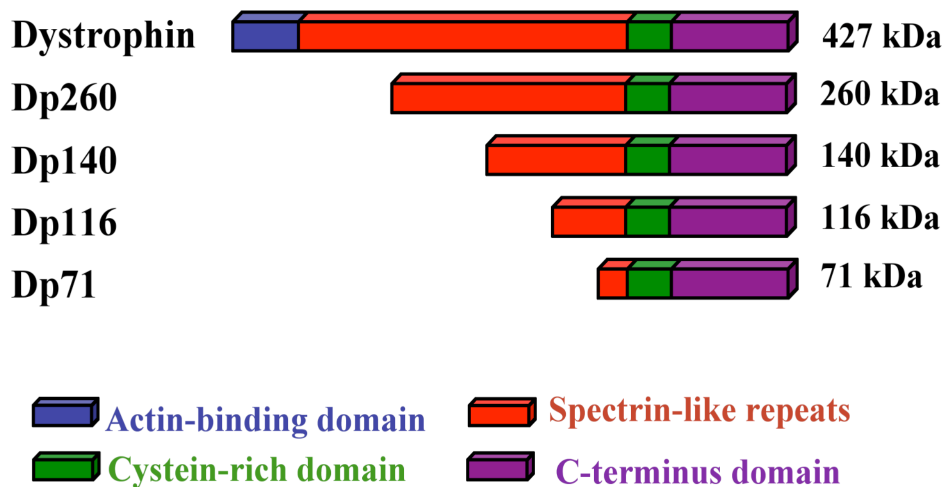


Fig. 3. Dystrophin isoforms and splice variants.

C. The dystrophin protein

Dystrophin is a member of the spectrin superfamily of proteins (Davison MD and Critchley DR, 1988), which includes the spectrins and the α-

actinins. Based on sequence prediction, similarities to other proteins and protein-binding capabilities the dystrophin molecule can be organized into four structural domains: the amino-terminal actin-binding domain, a central rod domain, a cysteine-rich domain, and a carboxy-terminal domain (Fig. 4).

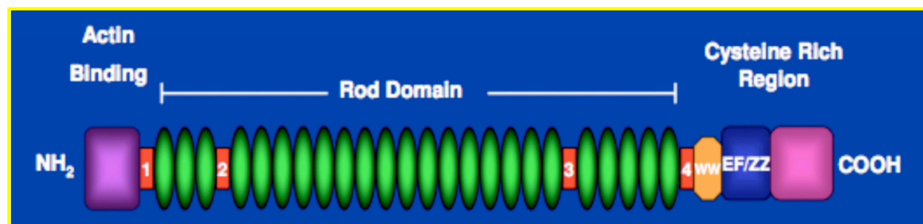


Fig. 4. The schematic organization of the dystrophin protein. The identifiable domains in the cysteine-rich region and COOH terminus (CYS) of dystrophin are identified. These are the WW domain, the EF hands, the ZZ domain and the paired coiled-coil (CC).

The amino acid terminus of dystrophin has homology with the amino-terminus of α-actinin and binds to actin filaments of the cytoskeleton. The rod domain accounts for the majority of the dystrophin protein and is composed of 24 spectrin like α-helical coiled-coil repeats interrupted by proline rich regions, which is thought to give a flexible rodlike structure similar to β-spectrin. At the end of the 24th repeat is the fourth hinge region followed by a WW domain. The WW protein-binding motif is found in a wide range of signalling, regulatory, and cytoskeletal proteins and binds to proline rich substrates (Einbond A and Sudol M, 1996). The cysteine-rich domain contains two putative "EF-hand" motifs that are similar to those in α-actinin and that could bind intracellular calcium and a single ZZ domain (Ponting CP et al, 1996). The ZZ domain contains a

number of conserved cysteine residues that are predicted to form the coordination sites for divalent metal cations. The ZZ domain of dystrophin binds to calmodulin in a Ca^{2+} -dependent manner (Anderson JT et al, 1996).

The carboxy-terminal of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain (Blake DJ et al, 1995). This region has been termed the coiled-coil domain (CC) forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other dystrophin-associated proteins (Sadoulet-Puccio HM et al, 1997).

D. Mutations in DMD

The frequency of DMD coupled with a high new mutation rate has led to the characterization of hundreds of independent mutations. There is some correlation between mutations in the *DMD* gene and the resulting phenotype. Approximately 65% of DMD and BMD patients have gross deletions of the *DMD* gene (Koenig M et al, 1989). After the characterization of many of such mutations, it became clear that the size and position of the deletion within the gene often did not correlate with clinical phenotype observed. This observation can be largely explained by the reading frame theory of Monaco (Monaco AP et al, 1988). This proposes that if a deletion leads to the expression of an internally truncated transcript without shifting the normal open reading frame, then a smaller, but functional version of dystrophin could be produced. This scenario would be consistent with a BMD phenotype (Fig. 5). If, on the other hand, the deletion creates a translational frameshift, then premature termination of translation will result in the synthesis of a truncated

unstable protein. This latter scenario is often associated with extremely low levels of dystrophin expression due to mRNA or protein instability and results in a DMD phenotype (Fig. 5).

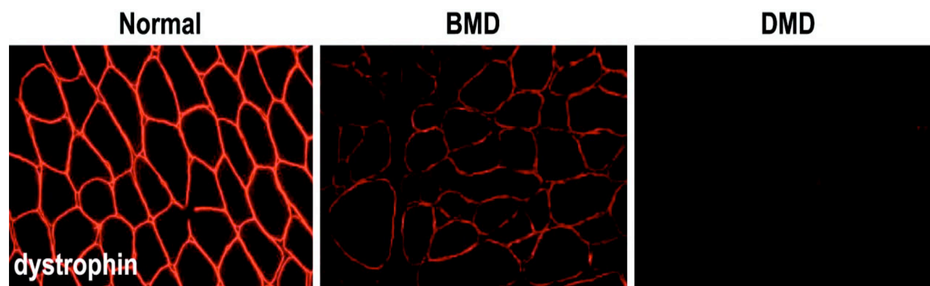


Fig. 5. Immunohistochemistry of dystrophin in human skeletal muscle from normal, BMD and DMD patients.

The vast majority of large deletions detected in BMD and DMD cluster around two mutation "hot spots" (Koenig M and Kunkel LM, 1990), although the reasons for this are unclear. It is possible; however, that the chromatin structure in Xp21 influence the occurrence of deletion or recombinant hotspots. Deletion cluster region I spans exons 45-53 (Beggs AH et al, 1990) and removes part of rod domain, while deletion cluster region II spans exons 2-20 and removes some or all of the actin-binding sites together with part of rod domain (Liechti-Gallati S et al, 1989). Most of the breakpoints occurring in cluster region II occur in the large introns 1 and 7. One-third of DMD cases are caused by very small deletions and point mutations, most of which introduce premature stop codons (Lenk U et al, 1993; Roberts RG and Sheng M, 2000). Unlike the large deletions that cluster in two regions of the *DMD* gene, small deletions and point mutations appear to be evenly distributed throughout the gene (Gardner RJ et al, 1995; Roberts RG et al, 1994). Although it

might be predicted that such mutations would give rise to normal amounts of truncated protein, usually very little or no protein is detected, indicating that the corresponding transcripts or the truncated proteins are unstable (Hoffman EP et al, 1987). At the NH_2 terminus of dystrophin, the importance of the actin-binding domain was demonstrated by the identification of a missense mutation (Arg for Leu-54) that resulted in DMD phenotype associated with reduced amounts of protein (Prior TW et al, 1995). Furthermore, DMD patients having in-frame deletions of exons 3-25 and producing normal amount of truncated protein have been described (Vainzof M et al, 1993).

The rod domain of dystrophin has been found to accommodate large in-frame deletions without serious clinical consequences. The most notable example was the discovery of a patient with an in-frame deletion of 46% of the dystrophin coding sequence, which resulted in a very mild case of BMD (deletion of exons 17-48) (England SB et al, 1990). This observation suggests that the rod domain acts as a spacer between the actin binding domain and the cystein-rich and COOH-terminal domains of dystrophin, and truncation of this region merely shortens the bridge between these two functional regions without adversely affecting the function of protein.

Although few missense mutations have been described in DMD patients, two informative substitutions have been identified in the cysteine-rich domain. The substitution of conserved cysteine residue with a tyrosine at position 3340 results in reduced but detectable levels of dystrophin. This mutation alters one of the coordinating residues in the ZZ domain that is thought to interfere with the binding of the dystrophin-associated protein β-dystroglycan (Lenk U et al, 1996). A small number of cases have been

reported in which an abnormally truncated protein that is deleted for the COOH terminus is synthesized and localized at the sarcolemma. A DMD patient was found to have a deletion that removed almost the entire cysteine-rich and COOH-terminal domain (Bies RD et al, 1992; Hoffman EP et al, 1991). The abnormal protein was normally localized but resulted in a severe clinical phenotype.

E. Dystrophin-associated protein complex (DAPC)

Dystrophin is located beneath the muscle sarcolemma associated with a membrane-spanning protein complex that connects the cytoskeleton to the basal lamina. The protein complex associated with dystrophin is of fundamental importance in the pathogenesis of the muscular dystrophy, since mutations in many components of the dystrophin-associated protein complex (DAPC) cause other forms of autosomally inherited muscular dystrophy (Tab. 1). The dystrophin complex is monomeric (Rybakova IN and Ervasti JM, 1997) it spans the membrane and links the actin-based cytoskeleton to the muscle basal lamina (Fig. 6). Thus the DAPC can be thought of as a scaffold connecting the inside of a muscle fiber to the outside. The DAPC can be divided into several separate sub-complexes based on their location within the cell and their physical association with each other. Using detergent extraction and two-dimensional gel electrophoresis, Yoshida et al (Yoshida M and Ozawa E, 1990) showed that the DAPC could be dissociated into three distinct complexes. These complexes are the 1) the dystroglycan complex, 2) the sarcoglycans-sarcospan complex, and 3) the cytoplasmic, dystrophin containing complex.

Disease	Inheritance pattern	Locus	Gene product	OMIM number
Dystrophinopathies				
Duchenne/Becker MD	Chromosome X	Xp21.2	Dystrophin	310200
Emery Dreifuss MD	Chromosome X	Xq28	Emerin	310300
Limb girdle MD (LGMD)				
Type 1A	Autosomal dominant	5q31	Myotilin	159000
Type 1B	Autosomal dominant	1q21.2	Laminin A/C	159001
Type 1C	Autosomal dominant	3p25	Caveolin 3	601253
Type 1D	Autosomal dominant	7q	Not known	603511
Type 1E	Autosomal dominant	7q	Not known	603511
Type 2A	Autosomal recessive	15q15.1-q21.1	Calpain 3	253600
Type 2B	Autosomal recessive	2p13.3-p13.1	Dysferlin	253601
Type 2C	Autosomal recessive	13q12	γ-Sarcoglycan	253700
Type 2D	Autosomal recessive	17q12-q21.33	α-Sarcoglycan	600119
Type 2E	Autosomal recessive	4q12	β-Sarcoglycan	600900
Type 2F	Autosomal recessive	5q33	δ-Sarcoglycan	601287
Type 2G	Autosomal recessive	17q12	Telethonin	601954
Type 2H	Autosomal recessive	9q31-d34.1	E3-ubiquitin ligase	254110
Type 2I	Autosomal recessive	19q13.3	Fukutin-related protein	606596
Type 2J	Autosomal recessive	2q24.3	Titin	Not available
Congenital muscular dystrophy (CMD)				
Merosin positive				
Rigid spine syndrome	Autosomal recessive	1p36-p35	Selenoprotein N1	602771
Ullrich syndrome	Autosomal recessive	21q22.3	Collagen VI subunit α2	254090
Merosin deficient without brain involvment				
Type 1A	Autosomal recessive	6q22-q23	α2-Laminin	156225
Type 1B	Autosomal recessive	1q42	Not konwn	604801
Type 1C	Autosomal recessive	19q13.3	Fukutin-related protein	606596
CMD with brain involvment				
Fukuyama CMD	Autosomal recessive	9q31	Fukutin	253800
Walker-warburg syndrome	Autosomal recessive	9q34	*O*-Mannosyltransferase	236670
Muscle-eye-brain disease	Autosomal recessive	1p34-p33	POMGNT1	253280

Tab. 1. Genetic classification of muscular dystrophies

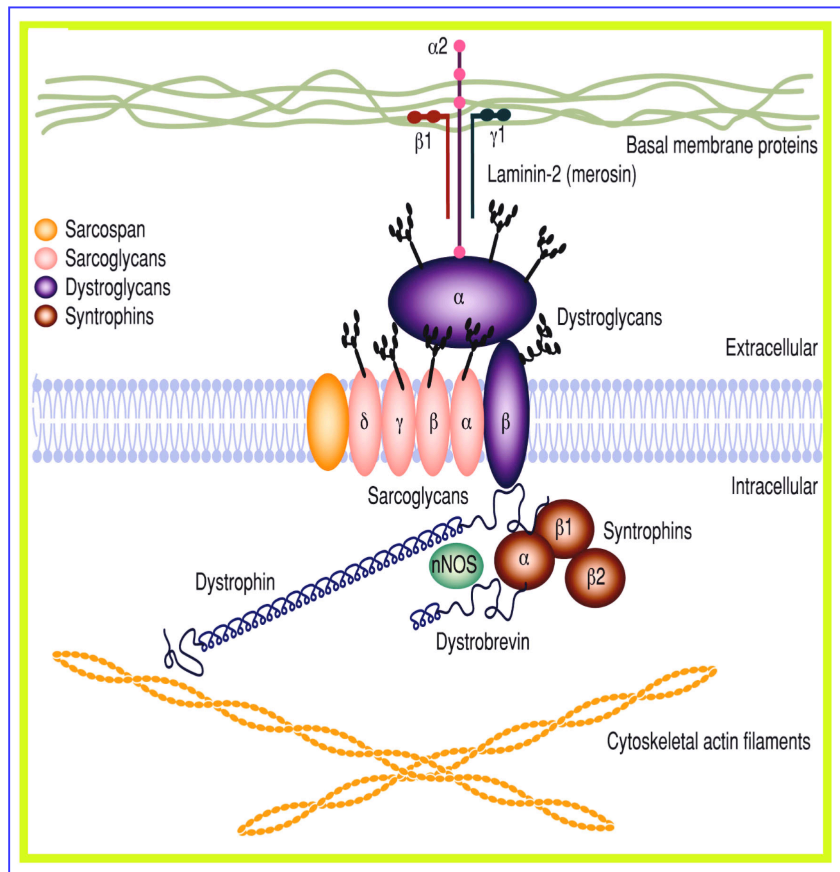


Fig. 6. The DAPC is composed of dystrophin, an elongated cytoskeletal protein that links to cytoplasmic actin filaments and the trasnmembreane components of the DAPC. Dystrophin binds to tha tail of β-dystroglycan. Dystroglycan is composed of two subunits, α and β, each produce from the some gene. Dystroglycan binds to the extracellular matrix protein laminin-α2. The sarcoglycan complex is composed of multilple subunits. Mutations in the genes encoding α-, β-, γ- and δ-sracoglycan lead to a similar phenotype as dystrophin mutations and include cardiomyophaty and muscular dystrophy in humans and mice. Additional subcomplexes in the DAPC in skeletal muscle include α and β dystrobrevin, the syntrophins and nNOS.

E.1 Dystroglycan complex

The single dystroglycan gene produces a precursor protein that is processed by an unidentified protease to produce α- and β-dystroglycan. The dystroglycan gene is composed of only two exons, and there is no evidence of alternative splicing, although several glycoforms are produced (Ibraghimov-Beskrovnaya O et al, 1992). In muscle, α-dystroglycan has a molecular mass of 156 kDa, whereas β-dystroglycan is 43 kDa. β-dystroglycan has a single transmembrane domain and is inserted into the muscle plasma membrane with the COOH terminus on the cytoplasmic side. In contrast, α-dystroglycan is located in the extracellular matrix (ECM) where it is thought to be directly associated with β-dystroglycan through multiple covalent interactions. The extreme COOH terminus of β-dystroglycan contains several proline residues that are required for dystroglycan binding to dystrophin (Jung D et al, 1995; Hasegawa M et al, 1999; Suzuki A et al, 1994; Suzuki A et al, 1992). The last 15 aminoacids of β-dystroglycan appear to bind directly to the cysteine-rich region of dystrophin. The structure of this region of dystrophin shows that dystroglycan forms contacts with both the WW domain and EF hands of dystrophin, emphasizing the functional importance of both of these domains to the dystrophin family of related proteins. β-dystroglycan also binds to Grb2, providing a known signalling pathway for β-dystroglycan (Yang B et al, 1995). Caveolin-3 also interacts with β-dystroglycan, and it may compete for the same binding site as dystrophin (Sotgia F et al, 2000). The amino-terminus of β-dystroglycan interacts with its extracellular binding partner α-dystroglycan. α-dystroglycan forms an important connection to the ECM

through its interaction with the α2 chain of laminin-2. It has been suggested that the interaction between α-dystroglycan and laminin-2 is dependent on the presence of anionic oligosaccharides on α-dystroglycan (Ervasti JM et al, 1997). Mutations in at least three different genes, fukutin, fukutin-related protein and LARGE have been shown to cause muscular dystrophy with abnormal α-dystroglycan processing (Aravind L and Koonin EV, 1999; Brockington M et al, 2001; Grewal PK et al, 2001). Thus it is tempting to hypothesize that the muscle disease in these patients is in part caused by the disruption of the laminin-2:α-dystroglycan interaction.

E.2 Sarcoglycans-Sarcospan Complex

In skeletal and cardiac muscle, the sarcoglycan complex is composed of four transmembrane glycoproteins α-, β-, γ- and δ-sarcoglycan and a member of the tetraspan family of proteins called sarcospan (Crosbie RH et al, 1997; Lim LE and Campbell KP, 1998). The distribution of α-sarcoglycan appears to be restricted to skeletal and cardiac muscle, whereas β-, δ- and γ-sarcoglycan are also expressed in smooth muscle (Barresi R et al, 2000; Roberds SL, et al, 1993). The molecular association of the sarcoglycan-sarcospan components with each other and with other components of the DAPC is currently unclear. Vainzof M et al (1999) suggest that α-, β- and δ-sarcoglycan might be closely associated with each other and that γ-sarcoglycan may interact with dystrophin. Mutations in the α-, β-, γ- and δ-sarcoglycan genes have been found to be primary defects in some forms of human autosomal-recessive limb-girdle muscular dystrophy (LGMD2) (Bushby KM, 1999). One feature of the sarcoglycanopathies is that the absence of one sarcoglycan has

important consequences for the stability of the other remaining sarcoglycan components at the plasma membrane. Typically, the loss of one sarcoglycan results in the absence or severe reduction in the remaining components of the sarcoglycan complex, although recent studies of patient muscle biopsies have demonstrated a variation in the pattern of sarcoglycan complex disruption (Bushby KM, 1999). Sarcospan is a member of thetetraspan family that associates tightly with the DAPC and sarcospan (Crosbie RH et al, 1997). Sarcospan is a highly hydrophobic protein whose amino and carboxyl-termini each face the cytoplasm. In addition, the presence of the sarcoglycan complex is required for the stability of sarcospan at the plasma membrane. Sarcospan-null mice maintain the proper assembly of the entire DAPC, showing normal muscle function and histology, serum creatine kinase levels, and impermeability of muscle fibers to Evan blue dye (Crawford GE et al, 2000).

E.3 The cytoplasmic complex

Dystrobrevin

The α- (DTNA) and β (DNTB)-dystrobrevin are members of the dystrobrevin family encoded by two different genes. The mammalian dystrobrevin genes encode several protein isoforms that are expressed in different tissues, including brain and muscle. The isoform expressed in muscle is α-dystrobrevin, while β-dystrobrevin is expressed in brain and other non-muscle tissues. DTNA has a high homology with the C-terminal domain of dystrophin. It is rich in cysteine residues. DTNA is able to bind with the sarcoglycan-sarcospan complex (Yoshida M et al, 2000). Blake DJ et al. (1996) found that the composition of the

dystrophin-associated protein complex in the brain differs from that in muscle. Because β-dystrobrevin and dystrophin are expressed in similar populations of neurons in the hippocampus and cortex, it is possible that β-dystrobrevin interacts directly with dystrophin. If this is the case, then β-dystrobrevin levels may be reduced in DMD patients similar to the reduction in sarcolemmal staining seen with other components of the DAPC in dystrophic muscle. The findings may be relevant to the cognitive dysfunction affecting DMD/BMD patients.

Syntrophin complex

The syntrophins are a heterogeneous multigene family of modular, 59 kDa cytoplasmic, phosphorylated, and adaptor proteins sharing a common domain structure. Syntrophins comprise five different proteins: one α (α-1, acidic), two β, (β-1 and β-2, basic) and two γ (γ-1 and γ-2). α-syntrophin is the major isoform of adult mouse skeletal muscle. The syntrophins (α, β-1 and β-2) bind directly to members of the dystrophin protein family: dystrophin, utrophin and α-dystrobrevin. The syntrophin complex binds to the second half of the C-terminal domain of dystrophin. Syntrophin also associates with neuronal nitric oxide synthase (nNOS). Based on their domain structure and association with neuronal nitric oxide synthase (Brenman JE et al, 1996), aquaporin (Adams V et al, 2001), ion channels (Gee SH et al, 1998) and kinases, syntrophins are thought to function as modular adaptors that recruit signalling proteins to the membrane via association with the DAPC.

Utrophin

In 1989, a parologue of dystrophin was identified on human chromosome 6 (Love DR et al, 1989). This dystrophin-related gene was later called utrophin because, in contrast to dystrophin, it is ubiquitously transcribed. Utrophin is slightly smaller than dystrophin, with an mRNA of 13 kb that corresponds to a protein of a predicted molecular weight of 395 kDa. The primary structure of utrophin is very similar to that of dystrophin, particularly in the N- and C- terminal ends that bind other proteins. The N-terminus of utrophin is similar to the actin-binding regions identified in dystrophin, spectrin and α-actinin. However, utrophin lacks the additional actin-binding activity associated with the dystrophin rod domain, and the N terminus of utrophin contains a short exstension, not found in dystrophin, which contributes to its affinity for actin. In view of the similarity in the primary structures of their C-termini, it is not surprising to find that utrophin binds members of the DAPC. Utrophin is widely expressed not only in skeletal, cardiac and smooth muscle cells, but also in vascular endothelia, retinal glial cells, platelets, Schwann cells of the peripheral nerves and several cell types within the kidney. In muscle fibers, utrophin is distributed very differently to dystrophin. In adult healthy muscle, utrophin is confined to the neuromuscular and myotendinous junctions. In contrast, in developing muscle, utrophin is found all along the sarcolemma, a distribution also seen in muscle regenerating after injury. The similarities of structure and binding partners between dystrophin and utrophin have raised the possibility of some functional redundancy between the two proteins. This hypothesis is further strengthened by the generation of several lines of mdx mice that harbour utrophin transgenes that can ameliorate the dystrophic

phenotype. In these mice, utrophin localizes throughout the sarcolemma and the DAPC is reconstituted. Even a modest two-threefold increase of utrophin over wild-type levels was found to be sufficient for some degree of morphological and functional recovery. These studies show that increasing utrophin levels over the normal adult levels significantly prevents pathology. The basis of the success of utrophin as surrogate for dystrophin comes from the ability of the former to play a structural role and restore the mechanical continuity between cytoskeletal actin, DAPC and the ECM (Khurana TS and Davies KE, 2003).

1.2 Animal models of muscular dystrophy

Several animal models manifesting phenotypes observed in specific genetic diseases have been identified in nature. In addition, a number of genetically engineered murine models for muscular dystrophy have been generated. These animals generally present physiological alterations frequently observed in human patients and can be used as important tools for genetic, clinic, and histopathological studies, providing important clues to the understanding of the pathogenesis of these disorders. Animal models are also very valuable for testing strategies for therapeutic approaches.

THE MDX MOUSE

The mdx mouse is the most widely used animal model for DMD. It was first described in 1984 because of the observation of elevated plasma levels of muscle creatine kinase and pyruvate kinase enzymes and histological lesions characteristic of muscular dystrophy. The mutants show mild clinical symptoms and are viable and fertile. Molecular analysis by Sicinski P et al. (1989) identified the mdx mutation as a single base substitution in exon 23 resulting in a premature stop codon in the mouse dystrophin gene, which leads to the absence of detectable dystrophin in the muscle, except in rare revertant myofibers (Hoffman EP et al, 1987). The absence of dystrophin also affects the expression of other DAPC components at the sarcolemma. Unlike DMD patients, the mdx mouse shows a mild non-progressive phenotype, associated to a

comparatively moderate muscle pathology, and muscle regeneration (Dangain J and Vrbova G, 1984). The mouse lives a relatively long life, although the lifespan is markedly shorter than that of normal C57/BL10 mice; the mdx mouse rarely lives past two years of age (Pastoret C and Sebille A, 1993), while wild-type mice live two and a half to three years (Lynch GS et al, 2001).

The muscle phenotype of mdx mice is similar to DMD, except for severity; the difference in severity is especially notable early in life. Mdx mouse muscles display a higher central nucleation index than DMD muscle (Karpati G et al, 1988), and show less fibrosis and less severe muscle degeneration in limb muscles. However, histopathologically there are many similarities between dystrophin-deficient myopathies in mdx mice and DMD patients. As in DMD, muscles in mdx mice undergo damage repair cycles, have increasing central nucleation with age, and show increasingly wider ranges of fiber size (Carnwath JW and Shotton DM, 1987, Coulton GR et al, 1988; Torres LF and Duchen LW, 1987). The damage is more extensive in DMD and has greater functional consequences. Fibrosis and adipose tissue deposits are less extensive in mdx muscle than in DMD muscle. Similarly to DMD, mdx mice undergo early degeneration/regeneration, which increases progressively. In DMD, however, there is more muscle to regenerate and the disease is more extensive, so the maximal regeneration capacity is reached earlier than in mdx mice. At this stage the fibrosis and fat infer more with regeneration in DMD tissue. Therefore, both mdx muscle and DMD muscle show disease progression, although at different rates.

Muscle fibers in mdx mice have a greater variation in cross-sectional area compared with the same muscles in C57/BL10 mice due to the

appearance of large hypertrophic and small regenerating fibers in the same muscle. There is also an increase in fibrosis with age (Anderson JE, et al. 1987). Mdx muscles have been shown to generate less twitch and titanic force per cross-sectional area than muscles in wild-type mice (Anderson JE, et al. 1987). The mdx diaphragm also exhibits a higher level of fibrosis than C57/BL10 mice, and fibrosis is also greater in mdx diaphragm than mdx limb muscles. The diaphragm muscle becomes dysfunctional prior to an increase in collagen content (Coirault C et al, 2003). The mdx model is said to have two limitations for the study of DMD; the first is its delayed progression compared to DMD, and the second is its milder tissue pathology and functional phenotype.

Mdx muscle and age

As mdx mice age, the muscles become more similar to those in DMD. Mouse muscles exhibit declining regeneration, decreasing overall weight and muscle weight, and large variations in fiber size, with many atrophic and split fibers (Pastoret C and Sebille A, 1995a, 1995b). There are also increases in the amount of fibrosis in skeletal muscle as the damaged myofibers are replaced with ECM and adipose tissue as part of tissue repair processes. In old mice, the progress of limb muscle weakness is quite advanced, and is accompanied by dystrophic changes in cardiac and respiratory muscles (Lefaucheur JP, et al. 1995). Kyphosis and spinal deformations progress with age in the mdx mouse (Laws N and Hoey A, 2004). Functionally, the diaphragm, and less so the skeletal muscles are able to generate lower force, and contractions are slower. As the mice age, they show progressive signs of exhaustion from exercise. After being subjected to involuntary exercise, older mdx mice were fatigued

and prostrated for several minutes, and some died shortly after exercise, while controls showed no signs of exhaustion (Vilquin JT et al, 1998).
With increasing age, mdx muscles show changes in protein expression. By three months of age proteins involved in energy metabolism, growth and differentiation, serine protease inhibition, calcium homeostatsis, and cytoskeleton organization are mostly overexpressed (Ge Y et al, 2004).

THE DYSTROPHIN-DEFICIENT DOG

Several dystrophin-deficient dogs have been identified and the causative genetic lesion defined in at least three (Schatzberg SJ et al, 1999; Sharp NJ et al, 1992; Winand NJ et al, 1994). The best-characterized phenotype is the golden retriever (the GRMD dog) (Cooper BJ et al, 1988). Muscle weakness becomes apparent at 2 mo and progress, life span is significantly reduced (Valentine BA et al, 1992). Histologically muscle shows necrosis, fibrosis and regeneration (Valentine BA et al, 1990). The GRMD dog shows perhaps the closest similarity to DMD and has been used to test potential treatments (Bartlett RJ et al, 2000; Sampaolesi M et al, 2006).

THE DYSTROPHIN-DEFICIENT CAT

Hypertrophic feline muscular dystrophy (HFMD) occurs in cats harbouring a deletion of the dystrophin muscle and Purkinje promoters, muscle levels of dystrophin are therefore much reduced (Gaschen FP et al, 1992; Winand NJ et al, 1994). Animals have an abnormal gait and histologically necrosis is present but fibrosis is not seen and hypertrophy is very marked. This latter feature causes death in some individuals. Although this odd phenotype could be due to the particular mutation, a

previous less well-characterized dystrophin-deficient cat also showed prominent hypertrophy, suggesting that this may be a feature of feline pathophysiology (Carpenter JL et al, 1989).

1.3 Skeletal muscle

ADULT SKELETAL MUSCLE CHARACTERISTICS

The muscle fibers are the basic contractile units of skeletal muscles. They are individually surrounded by a connective tissue layer and grouped into bundles to form a skeletal muscle. As well as being rich in connective tissue, skeletal muscles are highly vascularized to provide essential nutrients for muscle function. As the myofiber matures, it is contacted by a single motor neuron and expresses characteristic molecules for contractile function, principally different myosin heavy chain isoforms and metabolic enzymes. Both the motor neuron and the myoblast origin have been implicated to play a role in specifying the myofiber contractile properties, although the precise mechanisms remain to be defined. Nevertheless, individual adult skeletal muscles are composed of a mixture of myofibers with different physiological properties, ranging from a slow-contracting/fatigue-resistant type to a fast-contracting/non-fatigue-resistant type. The proportion of each fiber type within a muscle determines its overall contractile property. Thus the functional properties of skeletal muscle depend on the maintenance of a complex framework of myofibers, motor neurons, blood vessels, and the extracellular connective tissue matrix (Fig. 7).

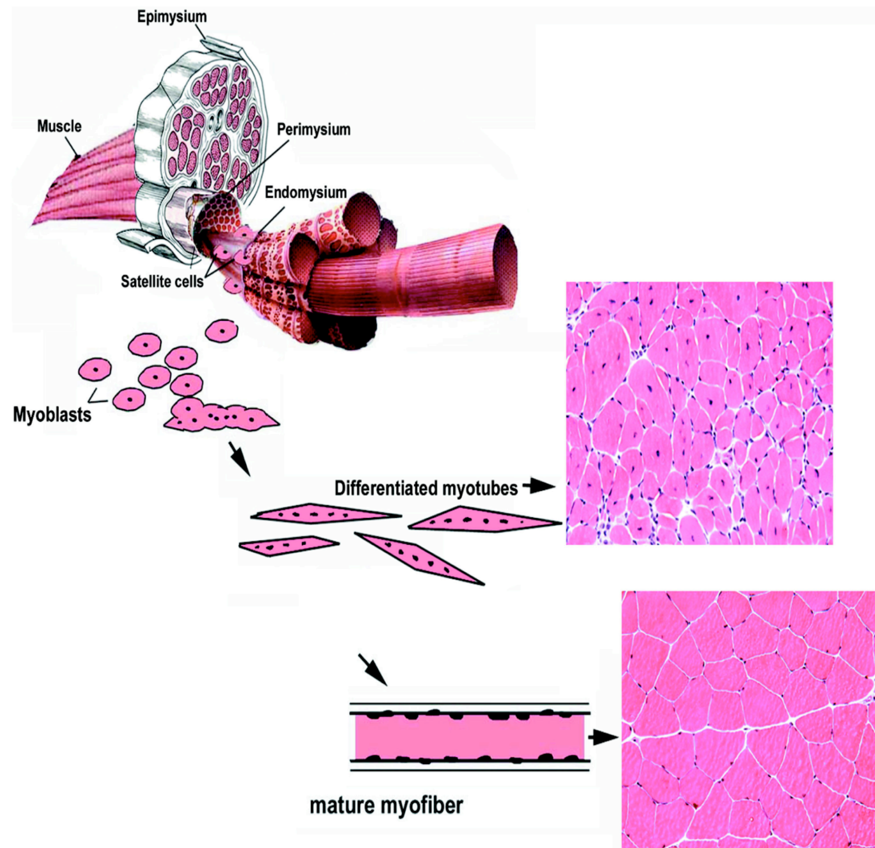


Fig. 7. Schematic drawing of the structural design of skeletal muscle. The endomysium is the connective-tissue layer that surrounds individual myofibers, the perimysium surrounds fascicles or bundles of myofibers, and the epimysium is the outside connective-tissue layer that surrounds the skeletal muscle. On muscle injury, the satellite cells are released and are activated to become myoblasts, which eventually differentiate into immature (myotubes) and mature muscle fibers. The nuclei are located in the central portion of the myotubes (immature myofibers), but they eventually migrate to the periphery of the myofiber when the muscle fibers mature.

Morphological characteristics of skeletal muscle regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage. After many years of research, it has become clear that the process occurring in injured muscle (necrosis/degeneration, inflammation, repair, and scar-tissue formation) are all interrelated and time-dependent.

Muscle regeneration process

Skeletal muscle is a dynamic tissue that is capable of responding to physiological stimuli or a severe injury by mounting a well-orchestrated regenerative response that restores the cytoarchitecture within a 2-week period (Hawke TJ and Garry DJ, 2001). The capacity for this regenerative response is primarily due to a mononuclear cell population termed satellite cells. These satellite cells were initially characterized as small, mononuclear cells that occupied a peripheral position in relation to the adjacent, larger multinucleated myofiber (Mauro A, 1961).

In response to injury or disruption of the basal lamina, the satellite cells become activated and have a remarkable proliferative capacity. Ultimately, the satellite cells either fuse to form multinucleated myotubes or re-establish a residual pool of quiescent satellite cells that have the capability of supporting additional rounds of regeneration. However, this regenerative capacity is not unlimited, as exhaustion of the satellite cell population is an important factor in the deterioration and the demise of patients that have congenital myopathies such as DMD.

Muscle satellite cells

In the adult, satellite cells are mitotically quiescent and reside in a niche between the basal lamina and the sarcolemma of their associated muscle fibers. Following activation, satellite cell will leave their niche and move outside of the basal lamina. Start to cycle and coexpress Pax7 and MyoD. The descendants of activated satellite cells, the skeletal myoblasts, undergo multiple rounds of division and most of them will downregulate Pax7, express myogenin and differentiate to fuse and form multinucleated myofiber. A fraction of these myoblasts will maintain Pax7, loose expression of myogenic markers and eventually leave the cell cycle (Olguin HC and Olwin BB et al, 2004; Zammit PS et al, 2004) (Fig. 8).

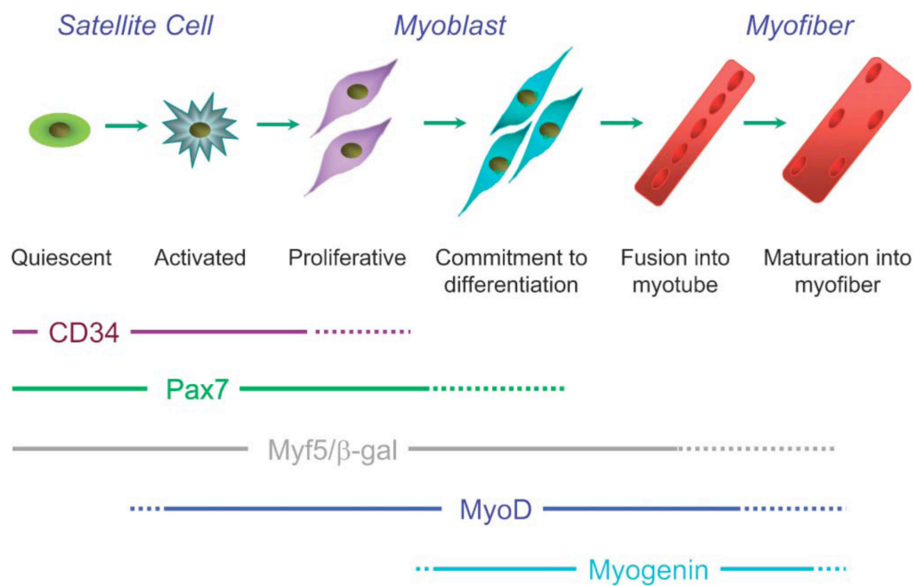


Fig. 8. Schematic rapresentation of satellite cell myogenesis and markers typical of each stage. Satellite cells are quiescent in normal adult muscle and

can be activated by muscle damage. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before committing to differentiation and fusing to form myotubes, which then mature into myofibers. CD34, Pax7 and Myf5/β-gal are expressed in quiescent satellite cells. Satellite cell activation is marked by the rapid onset of MyoD expression, whereas myogenin later marks the commitment to differentiation.

Activation of satellite cells requires the timely, controlled up-regulation of muscle transcription factors and muscle specific genes (Fig. 9). This process is regulated through mechanisms involving cell-cell and cell-matrix interactions as well as extracellular secreted factors. Muscle injuries have been shown to cause the release of biologically active molecules into the extracellular space. Different stimuli have been proposed as initiators of satellite cell activation; extract from the injured fibers, molecules released by the invading macrophages, and soluble factors from connective tissue have all been proposed (Hawke TJ and Garry DJ, 2001). In vitro studies have implicated an extensive number of trophic factors, including members of fibroblastic growth factor (FGF) and transforming growth factor-β (TGF-β) families, insulin growth factor (IGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) family of cytokines, and ATP, in maintaining a balance between growth and differentiation of satellite cells to restore a normal muscle architecture (Hawke TJ and Garry DJ, 2001).

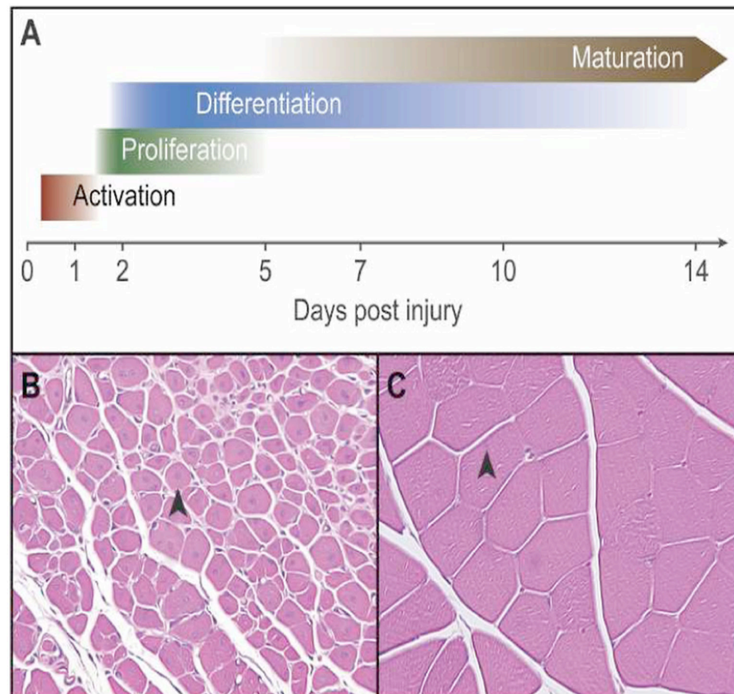


Fig. **9.** Muscle repair is characterized by discrete stages of regeneration. (A) Schematic outlining the stages of mdx muscle regeneration including the activation of satellite cells (within 2 h of injury), satellite cell proliferative stage, differentiation and maturation. (B) Following an intramuscular injection of cardiotoxin (a myonecrotic agent), ≈70-90% of the muscle is destroyed. Regeneration by satellite cells results in the formation of small, centronucleated myofibers. (C) Hematoxylin and eosin-stained transverse section of post-injured skeletal muscle reveals restoration of the cellular architecture within 2 weeks of injury. The nucleus occupies a peripheral position in fully mature myofibers.

Necrosis

The initial event of muscle degeneration is necrosis of the muscle fibers (Fig. 10). This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability.

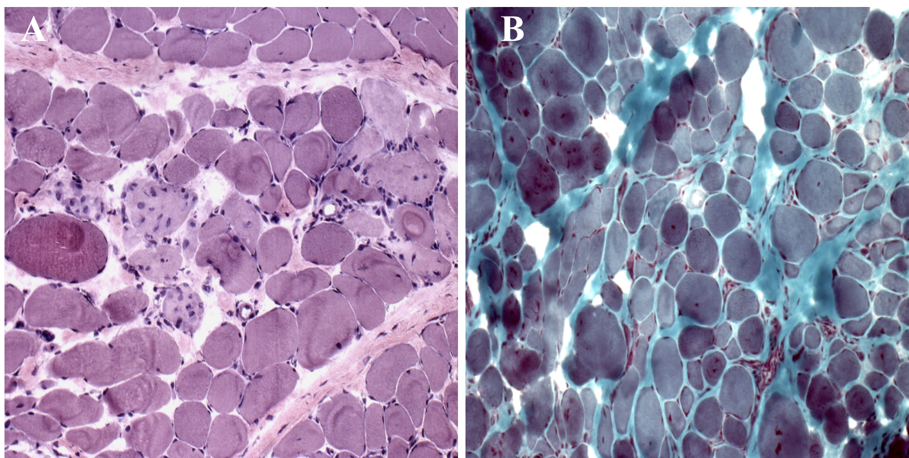


Fig. 10. Hematoxylin and eosin (A) and Gomori's trichrome (B) staining of DMD muscle section.

Increased serum levels of muscle proteins, such as creatine kinase, which are usually restricted to the myofiber cytosol, reflect disruption of the myofiber integrity. In human and animal models, increased serum creatine kinase is observed after mechanical stress and in the course of muscle degenerative diseases such as muscular dystrophies, all of which are characterized by the induction of a muscle regeneration process (Sorimachi H et al, 1997; Zatz M et al, 1991). Reciprocally, the uptake of low-molecular weight dyes, by the myofiber is a reliable indication of sarcolemmal damage and is also associated with strenuous exercise and muscle degenerative diseases (Hamer PW et al, 2002).

Impaired calcium homeostasis

It has been hypothesized that increased calcium influx after sarcolemmal or sarcoplasmic reticulum damage results in altered calcium homeostasis and increased calcium-dependent proteolysis that drives tissue degeneration (Alderton JM and Steinhardt RA, 2000). Calpains are calcium-activated proteases that can cleave myofibrillar and cytoskeletal proteins and hence are implicated in the process (Kwak KB et al, 1993). Thus disrupted myofibers undergo focal or total autolysis depending on the extent of the injury.

Inflammation

The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells (Fig. 11). Neutrophilis are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as 1-6 h after myotoxin or exercise-induced muscle damage (Orimo S et al, 1991). After neutrophil infiltration and 48 h postinjury, macrophages become predominant inflammatory cell type within the site of injury (Tidball JG, 1995). Macrophages infiltrate the injured site to phagocytose cellular debris and may affect other aspects of muscle regeneration by activating myogenic cells (Merly F et al, 1999; Roberts P et al, 1997). Thus muscle fiber necrosis and increased number of nonmuscle mononucleated cells within the damaged sire are the main histopathological characteristics of the early event following muscle injury.

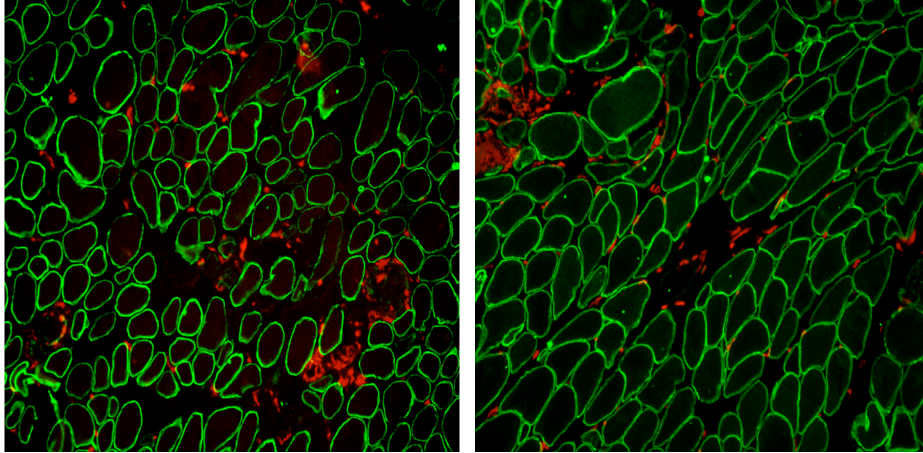
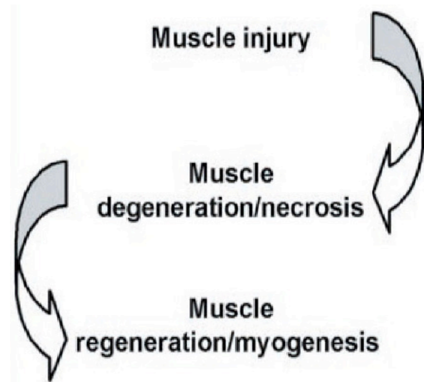


Fig. 11. Immunostaining of DMD muscle sections: anti-caveolin-3 (green), anti-macrophages (red).

Regenerative phase

Muscle degeneration is followed by the activation of muscle repair process (Fig. 12). Cellular proliferation is an important event necessary for muscle regeneration. Notably, the expansion of myogenic cells provides a sufficient source of new myonuclei for muscle repair (Grounds MD et al, 2002; Hawke TJ and Garry DJ, 2001). Following this myogenic proliferation phase, new muscle fibers are formed; myogenic cells differentiate and fuse to existing damaged fibers for repair or to one another for new myofiber formation (Snow MH, 1977; Snow MH, 1978). Newly formed myofibers are often basophilic (reflecting high protein synthesis) and express embryonic/developmental forms of myosin heavy chain (reflecting de novo fiber formation)(Whalen RG et al, 1990).



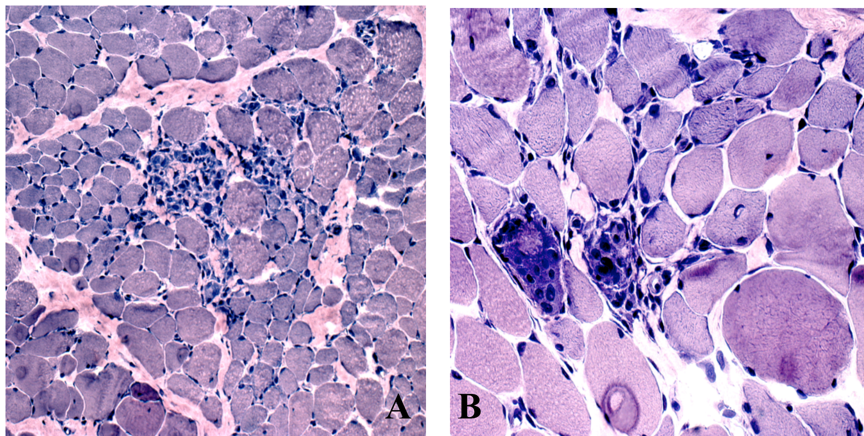


Fig. 12. Skeletal muscle repair process: mammalian skeletal muscle repair process is characterized by a degenerative phase followed by a regenerative phase. Hematoxylin and eosin staining: (A) 20x, (B) 40x, results in the rapid necrosis of myofibes and the activation of an inflammatory response leading to the loss of muscle architecture. Myofiber regeneratgion is characterized by the activation of myogenic cells to proliferation, differentiate, and fuse to necrotic fibers for repair or to each other for new fiber formation. Regenerating fibers are characterized by their small calibre and their centrally located myonuclei.

On muscle longitudinal sections and in isolated single fibers, central myonuclei are observed in discrete portions of regenerating fibers or along the entire new fiber, suggesting that cell fusion is not diffuse during regeneration but rather focal to the site of injury (Blaveri K et al, 1999). Fiber splitting is commonly observed in muscles from patients suffering neuromuscular diseases, in hypertrophied muscles, and in aging mouse muscles, all of which are associated with abnormal regenerative capacity (Bockhold KJ et al, 1998; Charge SB et al, 2002). Once fusion of myogenic cells is completed, newly formed myofibers increase in size, and myonuclei move to the periphery of the muscle fiber. Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from undamaged muscle.

1.4 The extracellular matrix

EXTRACELLULAR MATRIX

ECM is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM is composed of 3 major classes of biomolecules:

- **Structural proteins**: collagen and elastin
- **Specialized proteins**: fibronectin and laminin
- **Proteoglycans**: composed of protein core to which are attached long chains of repeating disaccharide units termed of glycosaminoglycans (GAGs) forming extremely complex high molecular weight components of the ECM
- **Functional components**: matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

The ECM provides essential functions, including structural support, cellular adhesion, and barrier to the movement of fluid and macromolecules. Each tissue type has particular matrix assemblies that contribute to specialized tissue functions. These specialized tissue functions are accomplished by particular extracellular matrix proteins.

- **Structural proteins**

Basement membrane components include proteins such as collagens, laminin, and proteoglycans. Interstitial matrix components include

collagens I and III, fibronectin, and small leucine-rich proteoglycans (SLRP) such as decorin and biglycan (Fig. 13).

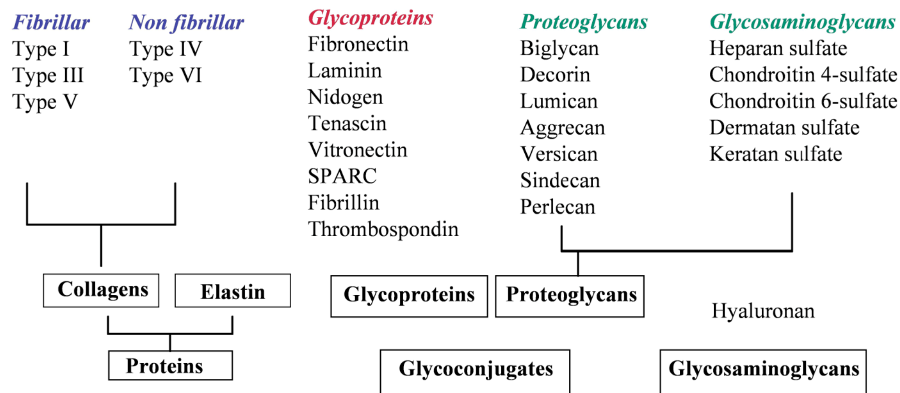


Fig. 13. Schematic representation of components of ECM.

Collagens

Collagens are the most abundant proteins found in mammalian. It is the major protein comprising the ECM. There are at least 30 different collagens genes dispersed trough the human genome. These 30 genes generate proteins that combine in a variety of ways to create over 20 different types of collagen fibrils. Types I, II and III are the most abundant and form fibrils of similar structure. Type IV collagen forms a two-dimensional reticulum and is a major component of the basal lamina. Collagens are predominantly synthesized by fibroblasts but epithelial cells also synthesize these proteins. The distinguishing feature of collagen is its chemical structure: a right-handed triple helix composed of three a-chains (Kuhn K, 1995). The three α-chains, either homotrimers or heterotrimers, are coiled around a central axis to form the triple helix (Fraser RD et al, 1983). Assembly of the helix takes place in the endoplasmic reticulum, and at this stage the molecule is referred to as a procollagen (Fig. 14).

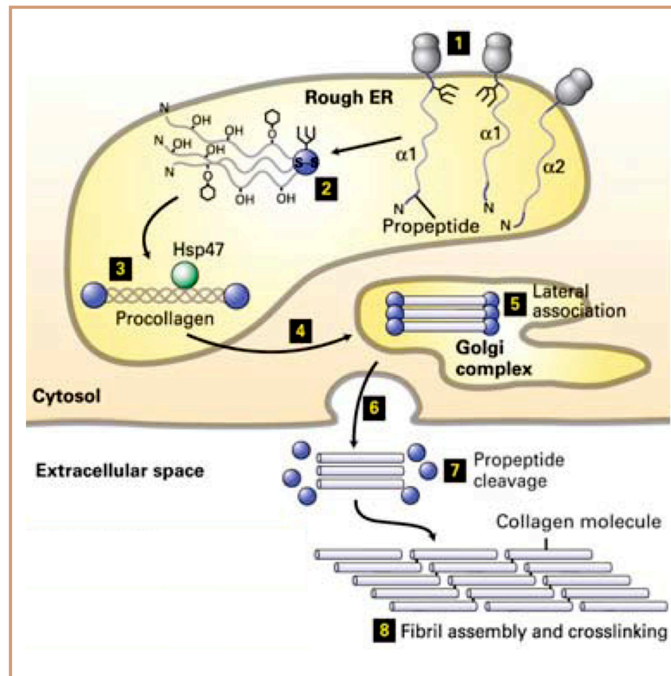


Fig. 14. Synthesis of collagen. At ER/Golgi pro-α-chains are produced, hydroxylated and glycosylated at selected Lys and Pro residues. Processed propeptides assemble into triple-helical pro-collagen. Disulphide bonds form between the N- and C-termini of procollagen. After exocytosis, N- and C-termini are trimmed, allowing fibril assembly.

Based on the structure and molecular organization of the three α-chains, collagen can be grouped into one of several classes. These classes are: fibril-forming collagen (I, II, III, V, XI), basement membrane collagen (IV), microfibrillar collagen (VI), anchoring fibrils (VII), hexagonal network-forming collagen (VIII, X), fibril-associated collagen with interrupted triple helices (FACIT collagens), transmembrane collagen

(XIII, XVII), and multiplexins (XV, XVI, XVIII) (Gelse K et al, 2003) (Tab.2).

TYPE	MOLECULAR COMPOSITION	TISSUE DISTRIBUTION
FIBRILLAR COLLAGENS		
I	$[\alpha1(I)]_2\alpha2(I)$	bone, skin, tendons, ligaments, cornea, internal organs (accounts for 90% of body collagen)
II	$[\alpha1(II)]_3$	cartilagine, vitreous humor
III	$[\alpha1(III)]_3$	skin, muscle, blood vessels
V	$[\alpha1(V)_2\alpha2(V)]$	cornea, teeth,bone, skin, smooth muscle
FIBRIL-ASSOCIATED COLLAGENS		
VI	$[\alpha1(VI)][\alpha2(VI)]$	most interstitial tissues
IX	$[\alpha1(IX)][\alpha2(IX)][\alpha3(IX)]$	cartilagine, vitreous humor
SHEET-FORMING and ANCHORING COLLAGENS		
IV	$[\alpha1(IV)]_2[\alpha2(IV)]$	all basal laminae
VII	$[\alpha1(VII)]_3$	below basal lamina of the skin
XV	$[\alpha1(XV)]_3$	widespread, near basal lamina in muscle
TRANSMEMBRANE COLLAGENS		
XIII	$[\alpha1(XIII)]_3$	hemidesmosomes in skin
XVII	$[\alpha1(XVII)]_3$	hemidesmosomes in skin

Tab. 2. Structure and function of collagens

1) Fibril-forming collagens

Fibril-forming collagens make up about 90% of the collagen in the body. They are divided into different sub-classes by the structure that they form in the body.

Collagens are synthesized as longer precursor proteins called procollagens. Collagen fibers begin to assemble in the endoplasmic reticulum (ER) and Golgi complex, the signal sequence is removed and numerous modifications take place in the collagen chains. Specific proline residues also are hydroxylated by prolyl 4-hydroxylase and prolyl 3-hydroxylase. Both prolyl hydroxylase are absolutely dependent upon vitamin C as co-factor. Following completion of processing the procollagens are secreted into the extracellular space where are converted to collagens by terminal propeptide cleavage actions by procollagen N-proteinase and procollagen C-proteinase (Hulmes DJ et al, 1995). The collagen molecules then polymerize to form collagen fibrils (Kuivaniemi H et al, 1991). At a later stage, strong covalent cross-linkages stabilize the collagen fibrillar structure (Seyedin SM and Rosen DM, 1990). Once collagen fibrils are formed they aggregate into large bundles called collagen fibers.

1A) Collagen type I

Collagen type I is a fibril-forming collagen. It is the most abundant collagen, and forms the bulk of the collagen found in most tissues. Collagen type I is a heterotrimer of two α1-chains and one α2(I)-chain (Von der MK, 1981).

1B) Collagen type III

Collagen type III, another fibril-forming collagen, is a homodimer of three α1(III)-chains. It is found in most collagen I-containing tissues with the exception of bone (Rossert JA et al, 1996). Collagen type III fibrils are often mixed with collage type I fibrils in muscle and other tissues. It is important to note that there is a need for collagen in muscle tissue, as it contributes to the physical properties of the tissue. However, excessive collagen build up in muscle and other tissues affects tissue function and can be sign of pathology.

Elastin

Elastin is the ECM protein primarily responsible for imparting elasticity to tissue. Elastin allows tissue to stretch and return to their original size without expending any additional energy. This protein is synthesized and secreted by fibroblasts and by smooth muscle cells. It is organized into elastic fibers, which consist of a core region of elastin proteins surrounded by a sheath of microfibril proteins

- **Specialized proteins**

Fibronectin

Fibronectin (FN) is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Fibronectin exists in two main forms: 1) as an insoluble glycoprotein dimer that serves as a linker in the ECM, and; 2) as a soluble disulphide linked dimer found in the plasma (plasma FN). The plasma form is synthesized by hepatocytes, and the ECM form is made by fibroblasts,

chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells. Fibronectin sometimes serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. FN also can serve to organize cellular interaction with the ECM by binding to different components of the ECM and to membrane-bound FN receptors on cell surfaces (Baron M et al, 1992).

Laminin

Laminin is a large (900 kDa) mosaic protein composed of many distinct domains with different structure and functions. Globular and rodlike domains are arranged in an extended four-armed, cruciform shape that is well suited for mediating between distinct sites on cells and other components of ECM. Laminin is the major non-collagenous component of the basal lamina. Structurally, the laminin protein is made up of three polypeptide chains- α, β and γ, giving it a total of six ends, accounting for a lot of its flexibility in connecting various molecules. They are a family of glycoproteins that are an integral part of the structural scaffolding in almost every animal tissue. Laminins are secreted and incorporated into cell-associated extracellular matrix. Laminins form independent networks and are associated with type IV collagen network via entactin and perlecan. They also bind to cell membranes through integrin receptors and other plasma membrane molecules, such as the dystroglycan glycoprotein complex. Through these interactions, laminins critically contribute to cell attachment and differentiation, cell shape and movement, maintenance of tissue phenotype, and promotion of tissue survival (Beck K et al, 1990).

Proteolgycans

Proteoglycans (PGs) are molecules composed of a specific core protein substituted with covalently linked glycosaminoglycan (GAG) chains. GAGs are linear, sulphated, negatively charged polysaccharides, which can be divided into two classes, namely (1.) sulphated GAGs comprising chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin, and heparan sulfate (HS), and (2.) non-sulfated GAGs such as hyaluronan (HA). GAG chains are made up of disaccharide repeating regions containing acetylated amino sugar moieties (N-acetyl-galactosamine or N-acetyl-glucosamine) and mainly uronic acid (D-glucoronic acid). The sulphated GAGs (CD, KS, DS, and HS) are linked to their respective protein cores via serine residues (Schaefer L and Schaefer RM, 2009)(Fig. 14).

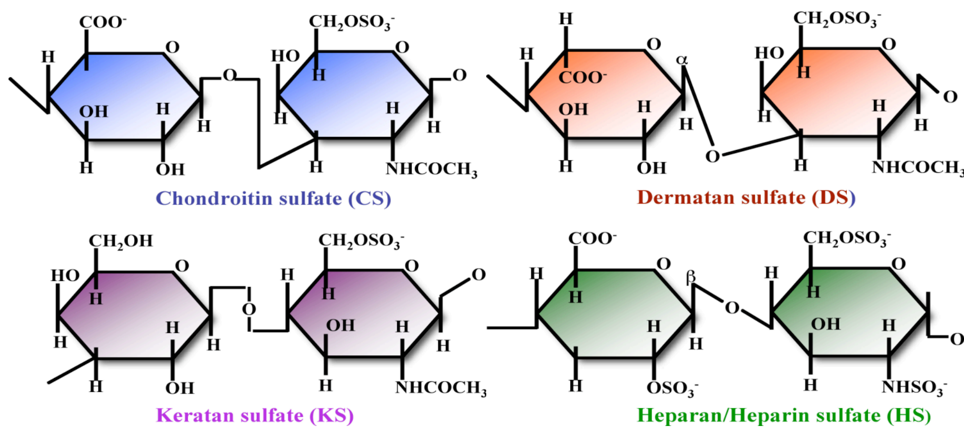


Fig. 14. The structure of sulphated GAGs (CD, KS, DS, and HS).

After synthesis, some PGs such as the small leucine-rich PGs (SLRP) and the large molecular weight PGs versican (CS/DSPG) and aggrecan (CS/KSPG) are secreted into the pericellular environment or are incorporated into basement membranes (Bi Y et al, 2005).
The enormous molecular diversity of PGs resulting from the various combinations of protein cores substituted with one or more GAG chains of various subtypes provides the structural basis for the multitude of their biological functions.
A comprehensive classification of PGs has been made according to the properties of their core proteins, their localization, size, and modular composition in addition to the type of GAG carried by the protein core. So, it is possible to identify three major PG families: (1.) SLRPs, (2.) modular PGs and (3.) cell-surface PGs.

(1.) Small leucine-rich proteoglycan (SLRP)

SLRPs are characterized by a protein core with leucine-rich repeats (LRRs), the presence of N-terminal cystein clusters and “ear repeats” (classes I-III), and at least one GAG side chain (Huxley-Jones J et al, 2007). Based on typical characteristics, such as conservation and homology at the protein and genomic level, cysteine-rich regions, and chromosomal organization, the family of SLRP is divided into five sub-families. The LRRs of the protein cores, which are considered to be particularly relevant for protein-protein interactions, have established SLRPs as important regulators of various biological processes (Brandan E et al, 2008) (Fig. 15).

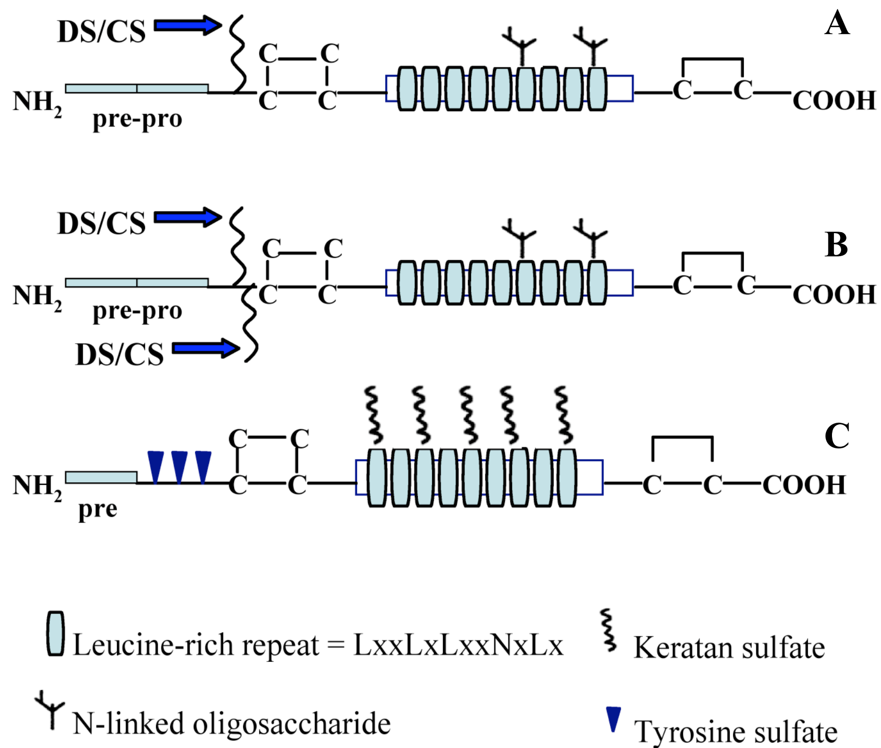


Fig. 15. Biglycan (A), decorin (B) and fibromodulin (C) structure. Pre: prepeptide; pre-pro: pre-propeptide.

Decorin is thought to bind to specific sites on collagen molecules as they assemble to prevent lateral association and thus may be responsible for collagen fibril diameter uniformity.
The binding site of decorin on type I collagen was identified near the C-terminus of type I collagen, very close to one of the major intermolecular crosslinking sites of collagen heterotrimers (Keene DR et al, 2000) (Fig. 16).

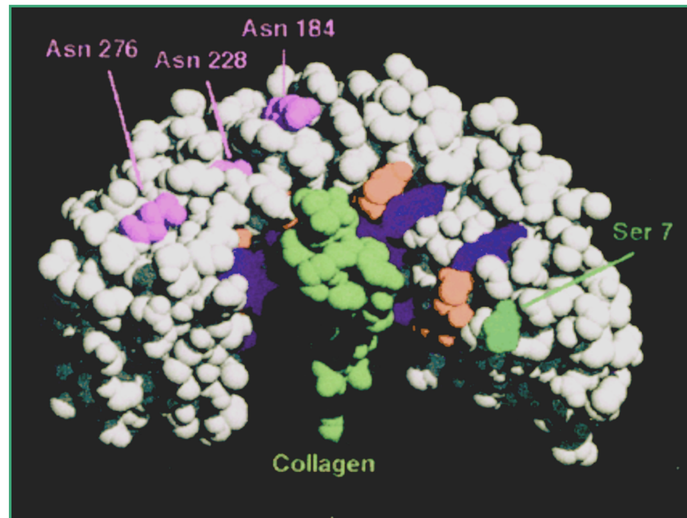


Fig. 16. Modeling of decorin structure. Decorin (in white) is predicted to bind collagen (green) within the groove of the predicted horseshoe-shaped strucuture (Weber I et al, 1996).

Decorin incorporation has been shown to increase tensile strength of uncrosslinked collagen fibers in vitro (Pins GD et al, 1997). Thus decorin could affect diameter uniformity, crosslinking, and mechanical properties of collagen fibrils. A mouse model for null-expression of decorin displays non-uniformity of connective tissue collagen fiber diameter, abnormal collagen organization, and skin fragility (Danielson KG et al, 1997). Collagen fibril uniformity may play an even more prominent role during wound healing. Decorin, biglycan and fibromodulin are capable of binding, albeit with different affinity, all three isoforms of TGF-β (Hildebrand A et al, 1994). Therefore, the biological implications of interacting with TGF-β, in order to develop anti-fibrotic therapies, have been investigated intensively. Several possible interactions of decorin with TGF-β have been postulated, such as direct binding and inactivation,

sequestration into the ECM via decorin bound to TGF-β (Kresse H and Schonherr E, 2001) and/or decorin-mediated interference with TGF-β signalling either through phosphorylation of Smad2 (Abdel-Wahab N et al, 2002) or LRP-1, a cell-surface receptor for decorin (Brandan E et al, 2008).

Whereas biglycan has been reported to have no effect on TGF-β-mediated fibrotic responses, decorin binding to TGF-β has been used to overcome TGF-β overproduction in different experimental models of fibrosis. Border et al. (1992) reported that administration of decorin inhibits increased ECM production and attenuates disease manifestations in the rat model of glomerulonephritis.

Introduction of decorin, by direct injection in hamster or by adenoviral gene transfer in mice reduces bleomycin-induced pulmonary fibrosis (Giri SN et al, 1997; Kolb M et al, 2001). Daily intraventricular injections of decorin have been used successfully to attenuate gliosis and inflammation induced in rat cerebral hemisphere by penetrating incisonal wounds (Logan A et al, 1999). Similarly, in their reproducible muscle laceration injury model in mice, Fukushima et al. (2001) showed that decorin injection into muscle prevents fibrosis in a dose-dependent way and enhances muscle regeneration, resulting in near-complete functional recovery. Li et al. (2004) recently demonstrated that in vitro overproduction of ECM proteins by myoblasts genetically engineered to express TGF-β, was prevented by decorin treatment.

Major functions of decorin, the prototype member of SLRPs are summarized in Tab. 3.

Major regulatory functions	Receptors
Collagen fibrillogenesis	
Tumor growth and metastatic spreading	EGF receptor
Angiogenesis	IGF-I receptor
TGF-β inhibition and sequestration	LRP-I
Renal and pulmonary fibrosis	IGF-I receptor
Muscular development and dystrophy	LRP-I
Fibrilin-I synthesis	IGF-I receptor
Wound healing	
Myocardial infarction	
Lyme disease	

Tab. 3. Decorin: biological functions and receptors (EGF epidermal growth factor, IGF-I insulin-like growth factor-I, LRP-I low-density lipoprotein receptor-related protein-I).

(2.) Modular PGs (CSPG-Lecticans)

The core proteins of lecticans share extensive structural similarity; they all have N- and C-terminal globular domains and a central domain of variable length with multiple sites for addition of chondroitin or dermatan sulfate chains and O-linked oligosaccharides.

Versican, aggregan, syndecan and betaglycan all belong to the lecticans family (Iozzo RV and Murdoch AD, 1996) (Fig. 17).

(3.) Cell-surface PGs

The cell-surface PGs include two major groups: (1.) membrane-spanning (syndecan) and (2.) GPI-linked PGs (glypicans). Syndecans and glypicans are the major heparan-sulfate PGs (HSPGs) on the cell surface with HS acting as co-receptor facilitating ligand encounters with signalling receptors (Bernfield M et al, 1999) (Fig. 17).

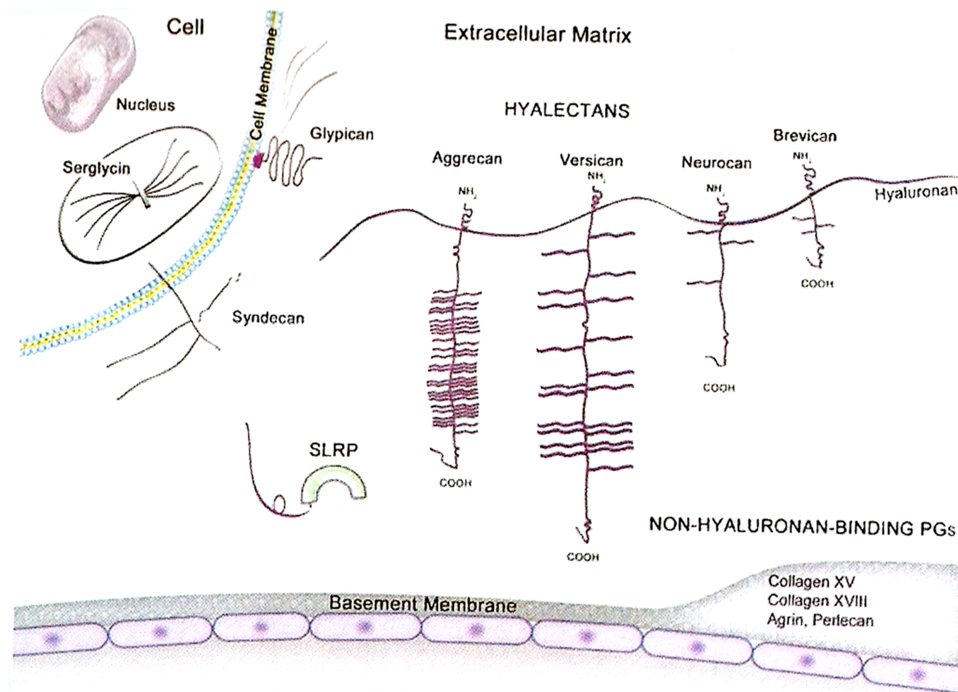


Fig. 17. Classification of proteoglycans (PGs) based on their location and binding. The heterogeneous group of PGs include those of the ECM, such as SLRP and modular PGs. Modular PGs are divided into hyalectans (hyaluronan- and lectin-binding PGs) and the non-hyaluronan-binding PGs of the basement membrane. The third group of cell-surface PGs encompasses mainly the membrane-spanning syndecans and the glycosylphosphatidylinositol-anchored glypicans. Serglycin is an intracellular PG found in hematopoietic and endothelial cells.

- **Functional components of ECM**

Matrix Metalloproteases (MMPs)

MMPs are a family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related. They are secreted in an inactive (latent) form, which is called a zymogen or a pro-MMP. These latent MMPs require an activation step before they are able to cleave ECM components. The activity of MMPs is regulated by several types of inhibitors, of whom the tissue inhibitors of metalloproteinases (TIMPs) are the most important. The balance between MMPs and TIMPs is largely responsible for the control of degradation of ECM proteins. MMPs are involved in the remodelling of tissue during embryonic development, cell migration, wound healing, and tooth development. However, a deregulation of the balance between MMPs and TIMPs is a characteristic of several pathological conditions, such as rheumatoid and osteoarthritis, cancer progression, and acute and chronic cardiovascular diseases (Snoek-van Beurden P and Von den Hoff J, 2005).

In the MMP family, at least 25 human members have been identified, and these enzymes are classified according to their substrate specificity and structural similarities. To be classified as an MMP, a protein must have at least the conserved prodomain and catalytic domain, which ligate the active site of Zn^{+2}. Additionally, MMPs have a flexible proline-rich hinge region and a haemopexin-like C-terminal domain, which functions in substrate recognition (with the exceptions of MMP-7, -23, and -26) (Fig. 18).

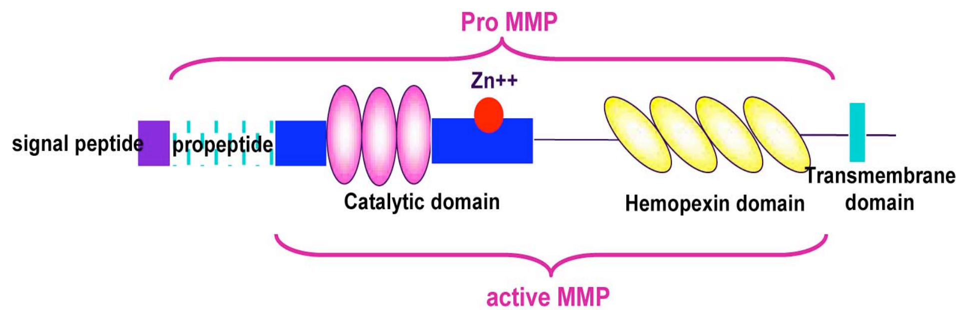


Fig. 18. Schematic of the domain structures of MMPs.

Six groups can be distinguished: (1.) The collagenase group includes MMP-1, MMP-8 and MMP-13. These are generally able to cleave the interstitial collagens I, II, and III. Collagenases are also able to digest certain other ECM and non-ECM proteins. (2.) The gelatinase group, which consists of MMP-2 and MMP-9, mainly digests gelatin, the denaturated form of collagen. (3.) The stromelysins, MMP-3 and MMP-10, digest ECM components such as collagen IV and fibronectin. (4.) The matrilysins, MMP-7 and MMP-26, digest several ECM components, such as fibronectin and gelatin. They lack the C-terminal hemopexin-like domain present in all other MMPs and are therefore also called the minimal domain MMPs. (5.) The membrane-type matrix metalloproteinases (MT-MMP), of which six forms are known, can digest a number of ECM proteins such as gelatin, fibronectin, and laminin. (6.) The remaining MMPs are gathered in a more heterogeneous subgroup because of their different substrate specificity, amino acid sequence, or domain organization. This group includes MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28, which cleave substrates such as elastin and aggrecan (Tab. 4).

Subgroup	MMP	Name	Substrate
1. Collagenases	MMP-1 MMP-8 MMP-13	Collagenase-1 Collagenase-2 Collagenase-3	Col I, II, III, VII, VIII, X, gelatin Col I, II, III, VII, VIII, X, aggrecan, gelatin Col I, II, III, IV, IX, X, XIV, gelatin
2. Gelatinases	MMP-2 MMP-9	Gelatinase A Gelatinase B	Gelatin, Col I, II, III, IV, VII, X Gelatin, Col IV, V
3. Stromelysins	MMP-3 MMP-10 MMP-11	Stromelysin-1 Stromelysis-2 Stromelysin-3	Col II, IV, IX, X, XI, gelatin Col IV, laminin, fibronectin, elastin Col IV, fibronectin, laminin, aggrecan
4. Matrilysins	MMP-7 MMP-25	Matrilysin-1 Matrilysin-2	Fibronectin, lamin, Col IV, gelatin Fibrinogen, fibronectin, gelatin
5. MT-MMP	MMP-14 MMP-15 MMP-16 MMP-17 MMP-24 MMP-25	MT1-MMP MT2-MMP MT3-MMP MT4-MMP MT5-MMP MT6-MMP	Gelatin, fibronectin, laminin Gelatin, fibronectin, laminin Gelatin, fibronectin, laminin Fibrinogen, fibrin Gelatin, fibronectin, laminin Gelatin
6. Others	MMP12 MMP-19 MMP-20 MMP-21 MMP-23 MMP-27 MMP-28	Macrophage metalloelastase Enamolysin XMMP CMMP Epilysin	Elastin, fibronectin, Col IV Aggrecan, elastin, fibrillin, Col IV, gelatin Aggrecan Aggrecan Gelatin, casein, fibronectin Unknown Unknown

Tab. 4. The classification of MMPs

In healthy tissues, some MMPs such as MMP-7, MMP-19, MMP-24, MMP-25, and MMP-26 are expressed at low levels. Many of the other MMPs, such as MMP-1, MMP-3, MMP-9, MMP-10, MMP-11, and MMP-13 are absent or only marginally expressed in normal, healthy, resting tissues. During repair or remodelling processes and in diseased or inflamed tissues, MMP expression is often increased.

Activation of MMPs

MMPs are translated as zymogens and contain a signal sequence peptide for targeting to secretory vesicles. MMPs are secreted or anchored to the cell surface, thereby confining their catalytic activity to membrane proteins or proteins within the secretory pathway or extracellular space (Ra HJ and Parks WC, 2007).

ProMMPs are kept in a catalytically inactive state by interaction between the thiol of the conserved prodomain cysteine residue (Cys^{73}) and the zinc ion of the catalytic site, so that the prodomain covers the catalytic cleft, thereby barring an interaction with a protein substrate. The thiol-Zn^{2+} interaction must be disrupted for a proMMP to become catalytically active (Van Wart HE and Birkedal-Hansen H, 1990). The thiol- Zn^{2+} interaction can be broken by three mechanisms: (1) direct cleavage of the prodomain by another proteinases such as furin; (2) reduction of the free thiol by oxidants or non-physiological reagents such as alkylating agents, heavy metal ions, and disulphides (Springman EB et al, 1990); and (3) allosteric perturbation of zymogen anchored to other macromolecules, such as integrins and proteoglycans (Ra HJ and Parks WC, 2007) (Fig.19).

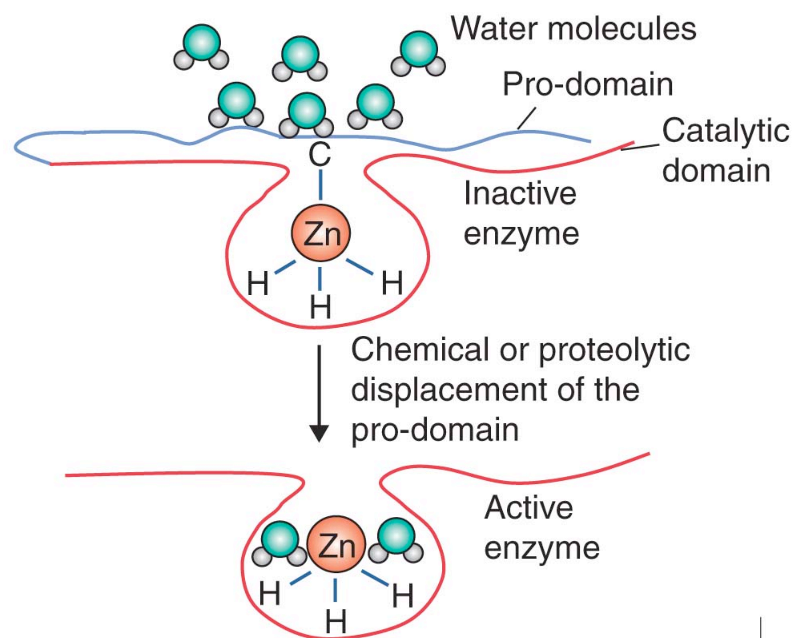


Fig. 19. The "cysteine-switch" mechanism regulating the MMP zymogens. The thiol group of conserved cysteine (C) at the carboxyl terminus of the pro-domain acts as a fourth inactivating ligand for the catalytic zinc atom in the active site; this results in the exclusion of water and keeps the enzyme latent. Displacement of the pro-domain by conformational change or proteolysis disrupts this cysteine-zinc pairing and the thiol group is replaced by water. The enzyme can then cleave the peptide bonds of its substrate.

Tissue Inhibitors of Matrix Metalloproteases (TIMPs)

TIMPs are small proteins with molecular weights between 21 and 28 kDa; these enzymes are codified by highly conserved genes and have overlapping function (Tab. 5). The mammalian TIMP family has four members, which share substantial sequence homology and structural

identity at the protein level. TIMPs have basically two structural domains: an N-terminal domain consisting of six conserved cysteine residues forming three disulfide loops, which possesses MMP-inhibitory activity, and a C-terminal domain that also contain six conserved cysteine residues and forms three disulfide loop (Tuuttila A et al, 1998).

	Molecular weight (kDa)	MMPs inhibited	Other functions	Location
TIMP-1	28	All MMPs ADAM10	Strong inihibitor of MMP-9	Secreted into ECM
TIMP-2	21	All MMPs	Forms trimolecular complex with pro-MMP-2 and MMP-14 to activate MMP-2	Secreted into ECM
TIMP-3	24 (unglycosylated) 27 (glycosylated)	All MMPs ADAM10 ADAM 17	Apoptosis, inhibit angiogenesis	Bound to the ECM
TIMP-4	22	All	Inhibits angiogenesis	Secreted into ECM

Tab. 5 Nomenclature, molecular weights, functions, and locations of TIMPs. (Baker AH et al, 2002)

By definition, all members of the TIMP family inhibit MMP activity. This is accomplished through coordination of the Zn^{+2} of the MMP active site by the amino and carbonyl groups of the TIMP N-terminal cysteine residue. TIMPs generally inhibit the activity of MMPs by the formation of a 1:1 complex. However, selective inhibition of some members of the MMP has been observed. For example, although TIMP-1 is the prototypic inhibitor for the most MMP family members, it is a poor inhibitor of the membrane-type MMPs (MT-MMP) and MMP-19. TIMP-3 inhibits members of the A Disintegrin And Metalloproteinase (ADAM) family of proteases, although the mechanism for this inhibition appears to be distinct from that of MMP inhibition. TIMP-2 is unique in that, in addition to

inhibiting MMP activity, it selectively interacts with MT1-MMP to facilitate the cell-surface activation of pro-MMP-2 by MT-MMP (Stetler-Stevenson WG, 2008) (Fig. 20).

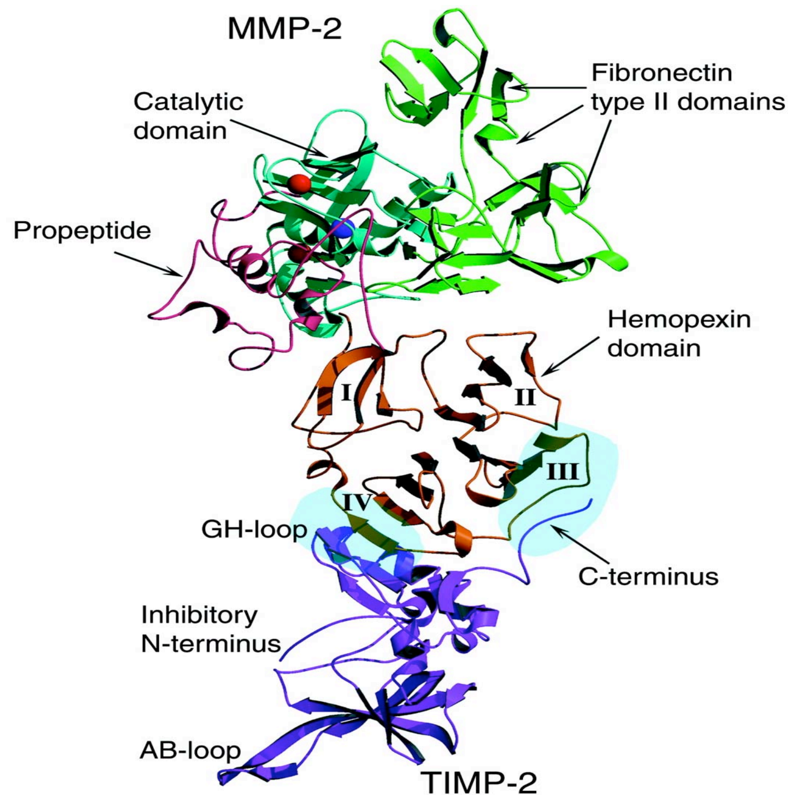


Fig. 20. Structure of the proMMP-2/TIMP-2 complex. Overall conformation: the proteinase and inhibitor interact via their C-terminal domains. The catalytic site of MMP-2 and the inhibitory active site of TIMP-2 are turned away from each other. This topology excludes an inhibitory interaction between the proteinase and inhibitor and implies that both proteins remain fully functional in the complex. Catalytic and structural Zn^{2+} ions are colored red and Ca^{2+} ion purple. The β-propeller blades of the hemopexin domain are numbered from I to IV. Two

light blue ellipsoids in blades III and IV indicate two areas of interaction between proMMP-2 and TIMP-2 molecules.

TIMP-3 is the only TIMP bound to the ECM it specifically interacts with sulphated glycosaminoglycans and as a result is sequestered in the ECM (Yu WH and Woessner JF, 2000), whereas the other TIMP family members remain soluble and diffusible.
TIMP-3 inhibits several membrane-bound molecules with sheddase functions, such as MMP-14, MMP-3 and TACE, indicating that TIMP-3 plays a central part in several important reactions, including cellular growth, cellular death, and tissue repair. By comparison relatively few studies have focused on TIMP-4, so less is known about this member of the TIMP family (Stetler-Stevenson WG, 2008).

MMP and TIMP regulation

Control over MMP and/or TIMP activity in vivo occurs at different levels and involves factors such as regulation of gene expression, activation of zymogens and inhibition of active enzymes by specific inhibitors. Many MMPs and TIMPs are regulated at the level of transcription by a variety of growth factors, cytokine and chemokines. While it is well recognized that MMP gene expression is mainly regulated at the transcriptional level, recent evidence, however, suggests that post-transciptional mechanisms are also involved in the control of MMP expression in response to certain cues. As an example, TGF-β1 increases MMP-2 and -9 levels, mainly by extending the half-life of MMP mRNAs in human gingival fibroblasts and prostate cancer cells (Overall CM et al, 1991); but induces MMP-13 expression by both inducing transcription and stabilizing the transcript in

mouse osteoblasts and fibroblasts (Delany AM and Canalis E, 2001; Yan C and Boyd DD, 2007).

Numerous pathological conditions are caused by the disruption of the balance between MMPs and TIMPs. Overproduction of MMPs is associated with tissue destruction in chronic inflammatory disease such as rheumatoid and osteoarthritis. In both conditions, it appears that the erosion of the cartilage is caused by an increased expression of MMPs such as MMP-1, MMP-8, and MMP-13 (Konttinen YT et al, 1996). MMPs are also involved in tumor growth and metastasis. In almost all human cancers, the MMP expression and activity are increased. The increased levels of MMPs seem to correlate with invasiveness and poor prognosis (Coussens LM and Werb Z, 2002). Deregulation of MMPs has also been implicated in distinct acute and chronic cardiovascular diseases, such as atherosclerosis, myocardial infarction, and heart failure (Galis ZS and Khatri JJ, 2002). Many different MMPs are involved in normal tissue remodelling but also play a central role in tissue resorption in pathological conditions.

1.5 Mechanisms of fibrogenesis

Normal wound healing

The Wound Healing Process is defined by the Wound Healing Society as "a complex and dynamic process that results in restoration of anatomic continuity and function". The wound healing process is a cascade of events, beginning with injury to tissue. Appropriate wound management is dependent on correct understanding of the normal repair process, the factors affecting this process and the interventions that can impact either positively or negatively on the outcome. There are four phases of normal wound healing:

Vascular Response (Haemostasis)

- Vasoconstriction – within seconds, regardless of the source of injury, blood vessels constrict to stop bleeding and reduce exposure to bacteria.
- Platelets cluster together at the site of injury to form a 'clot'.
- Stimulation of the clotting cascade results in the cleavage of fibrinogen by thrombin to form a fibrin plug, which together with fibronectin, holds damaged tissues together and provides a provisional matrix for the recruitment of inflammatory cells and later the migration of fibroblasts and other resident cells.
- Wound healing begins within minutes after tissue damage.

Inflammatory Response (Inflammation)

- Neutrophils are the first and most numerous white blood cells to arrive at the injured site. As they degranulate and die, macrophages predominate, accumulating at the wound site following recruitment

from the circulation and from the resident population.

- Macrophages are vital for normal wound healing and if their infiltration is prevented, healing is impaired. They act in concert with neutrophils to phagocytose debris and invading pathogenic microorganisms and are a source of chemoattractants and growth factors including: platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TGF-β and tumor necrosis factor α (TNF-α). These factors are mitogenic and chemotactic for endothelial cells, which surround the wound and migrate towards its centre forming new blood vessels. Wound healing is significantly impaired without macrophages.
- Fibroblasts and other resident cells migrate along the fibrin lattice into wound. The dense population of macrophages, fibroblasts and neovasculature embedded within a loose matrix of fibronectin, collagen and hyaluronic acid is referred to as granulation tissue or the provisional matrix. Failure to resolve the inflammation can lead to chronic nonhealing wounds.

The Proliferative Phase (Granulation)

- Fibroblasts degrade the provisional matrix via MMPs and respond to cytokine/growth factors by proliferating and synthesizing new extracellular matrix to replace the injured tissue with a connective tissue scar. Fibroblasts produce and deposit large quantities of matrix proteins, predominantly types I and III collagen, which increase the tensile strength of the wound.
- Collagen is secreted reconstructing connective tissue. Vitamin C, zinc, oxygen and iron are required for this process. The nature of the matrix components in wounds changes with time. In the earliest stage,

excessive type III collagen deposition is seen but later type I collagen predominates. Collagen fibrils become tightly packed and stabilized by the formation of inter- and intra-molecular cross-links. This series of events occurs in most tissues including skin, lung, heart and bone. Uncontrolled matrix accumulation, often involving aberrant cytokine pathways, leads to excess scarring and fibrotic sequelae.

Maturation Phase (Remodeling)

- The remodelling phase, during which collagen is synthesized, degraded and reorganized is also cytokine-mediated. Degradation of fibrillar collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. MMPs not only degrade matrix components, but also functions as regulatory molecules by driving enzyme cascades and processing cytokines, matrix and adhesion molecules to generate biologically active fragments. TIMPs provide a natural counterbalance to the MMPs and disruption of this orderly balance can lead to excess or insufficient matrix degradation. The coordinated regulation of enzymes and their inhibitors ensures tight control of local proteolytic activity (Fig. 21).

PHASES OF WOUND HEALING

INJURY 1 — 4 — 20 — CLOSURE X DAYS

HAEMOSTASIS

- Damaged Vessels constrict to slow blood flow
- Platelets aggregate to stop
- Bleeding
- Leulocytes migrate into tissue to initiate inflammatory process

INFLAMMATION

- Neutrophils secrete chemicals to kill bacteria
- Macrophages engulf and digest foreign particles and necrotic debris
- Macrophages release engiogenic substances to stimulate capillary growth and the granulation process

PROLIFERATION

- Fibroblasts proliferate in the wound and secrete glycoproteins and collagen
- Epidermal cells migrate from the wound edge
- Granulation tissue is formed from macrophages, fibroblasts and new capillaries

REMODELLING

- Fibroblasts secrete collagen to strengthen wound
- Wound remodelling occurs to reorganise fibres
- Wound contracts increasing tissue integrity
- Epidermal cells grow over connective tissue to close wound

Fig. 21. Schematic rappresentation of wound healing process.

Mechanisms of fibrogenesis

Fibrotic diseases occur in a large variety of vital organs, and the process of fibrosis seems common to all tissues. In all of fibrotic reactions, the underlying cellular and molecular mechanisms involve leukocyte infiltration, the persistence of inflammation in the tissue, and the proliferation of cells with a myofibroblast phenotype. The different cell types participating to this process sustain production of growth factors, proteolytic enzymes, angiogenic factors, and fibrogenic cytokines, which together stimulate the deposition of connective tissue elements that

progressively destroy and remodel normal tissue architecture (Kisseleva T and Brenner DA, 2008).

A common and unique mechanism regulates fibrosis in various fibroproliferative diseases. In contrast to acute inflammatory reactions, which are characterized by rapidly resolving vascular changes, edema, and neutrophilic infiltration, pathogenic fibrosis typically results from chronic inflammatory reactions-defined as a responses that persist for several weeks or months and in which inflammation, tissue distruction, and repair processes occur simultaneously.

The mechanism of fibrotic process

When injuries occur, damaged epithelial and/or endothelial cells release inflammatory mediators that initiate an antifibrinolytic-coagulation cascade, which triggers formation of both blood clots and a provisional ECM. Platelets are exposed to ECM components, triggering aggregation, clot formation, and hemostasis. Next, platelet degranulation promotes vasodilation and increased blood vessel permeability, while stimulated myofibroblasts and epithelial and/or endothelial cells produce MMPs, which disrupt the basement membrane, allowing the efficient recruitment of inflammatory cells to the site of injury. Epithelial and endothelial cells also secrete growth factors, cytokines, and chemokines, which stimulate the proliferation and recruitment of leukocytes across the provisional ECM. Neutrophils are the most abundant inflammatory cell at the early stages of wound healing. When they degranulate and die, macrophages are recruited. During this initial leukocyte migration phase, the activated macrophages and neutrophils eliminate tissue debris, dead cells, and any invading organisms. They also produce cytokines and chemokines, which

amplify the wound-healing response. These factors are also mitogenic and chemotactic for endothelial cells, which surround the injury and form new blood vessels as they migrate toward its center. Subsequently, T cells become activated and secrete profibrotic cytokines such as IL-13 and TGF-β, which in turn further activate the macrophages and fibroblasts. Activated fibroblasts transform into α-SMA–expressing myofibroblasts as they migrate along the fibrin lattice into the wound. Myofibroblasts are derived from local mesenchymal cells or recruited from the bone marrow. Epithelial cells can also undergo epithelial-mesenchymal transition (EMT), providing a rich renewable source of myofibroblasts. Following activation, myofibroblasts promote wound contraction, the process in which the edges of the wound migrate toward the center. Finally, epithelial and/or endothelial cells divide and migrate over the basal layers to regenerate the damaged tissue, which completes the normal healing process. However, when repeated injury occurs, chronic inflammation and repair cause an excessive accumulation of ECM components (such as hyaluronic acid, fibronectin, proteoglycans, and interstitial collagens), which contribute to the formation of a permanent fibrotic scar (Fig. 22).

The net amount of collagen deposited by fibroblasts is regulated continuously by collagen synthesis and collagen catabolism. Various MMPs and their inhibitors, which are produced by granulocytes, macrophages, epidermal cells, and myofibroblasts, control the turnover of collagen and other ECM proteins. Shifts in these opposing mechanisms regulate the net increase or decrease of collagen within a wound. The expanding pool of mesenchymal cells further exacerbates the response. In the remodeling phase, the synthesis of new collagen by

fibroblasts exceeds the rate at which it is degraded so that the total amount of collagen continues to increase. Although inflammation typically precedes fibrosis, results from experimental models of this process have demonstrated that fibrosis is not necessarily driven by inflammation at all times, suggesting that the mechanisms that regulate fibrogenesis are, to a certain extent, distinct from those regulating inflammation. This might explain the general lack of efficacy of antinflammatory mediators in the treatment of fibrotic disease and the need to identify targeted antifibrotic therapies.

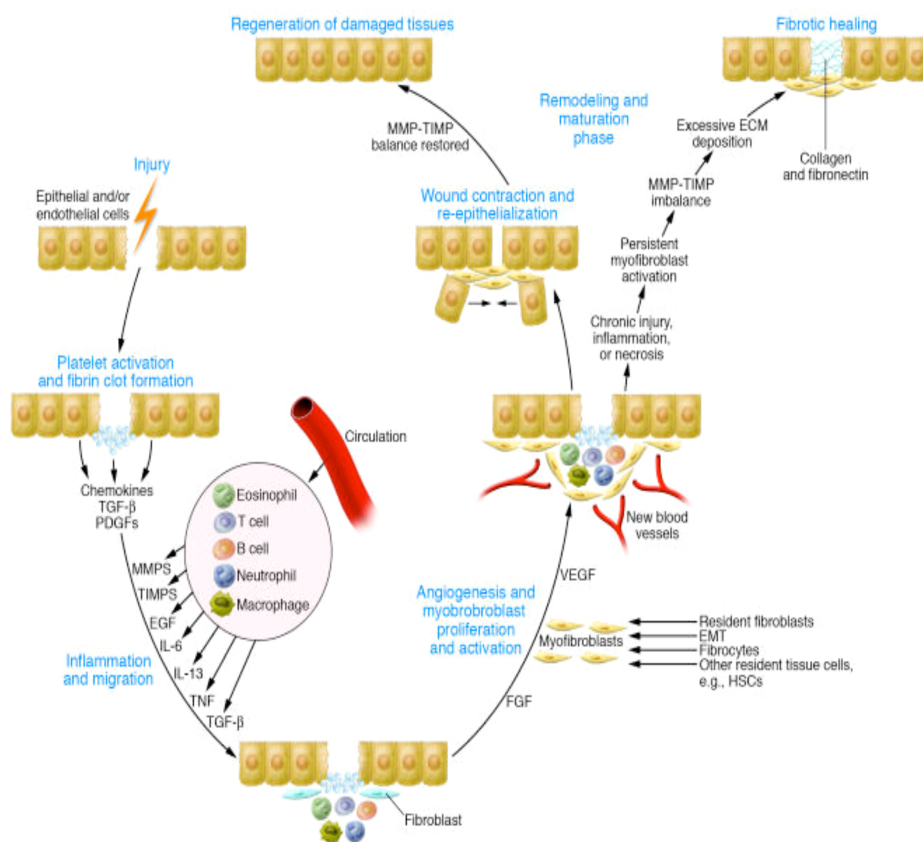


Fig. 22. These events include: 1) immediate damage to the ephitelial/endothelial barrier; 2) release of TGF-β1, the major fibrogenic cytokine; 3) recruitment of inflammatory cells; 4) induction of reactive oxygen species (ROS); 5) activation of collagen producing cells; 6) matrix activation of myofibroblast; and finally, 7) in the absence of continuous injury, reversal of fibrosis can occur.

Fibroproliferative diseases

Fibroproliferative diseases, including pulmonary fibrosis, systemic sclerosis, liver cirrhosis, cardiovascular disease and progressive kidney disease are a leading cause of morbidity and mortality and can affect all tissue and organ systems.

- **Interstitial lung diseases (ILDs)**

ILDs comprise a group of acute and chronic disorders with varying degrees of inflammtion and fibrosis. One of the most common is idiopathic pulmonary fibrosis (IPF). IPF is a progressive disease with a median survival of only 2 to 3 years following diagnosis. Histologically, IPF lungs have alternating regions of normal lung parenchyma, interstitial inflammation and fibrosis. These features are a result of failed alveolar reepithelialization, fibroblast persistence, and excessive deposition of collagen and other ECM components, leading to irreversible loss of lung function.

Different models of pulmonary fibrosis have been developed over the years. Most of them mimc some, but never all features of human IPF, especially the progressive and irreversible nature of the condition. Common methods include radiation damage, instillation of bleomycin, silica or asbestos, and transgenic mice. The standard agent for induction of experimental pulmonary fibrosis in animals is bleomycin. It causes inflammatory and fibrotic reactions within a short period of time; the

initial elevation of pro-inflammatory cytokines is followed by increased expression of pro-fibrotic markers (TGF-β1, fibronectin, procollagen-1) (Chaudhary NI et al, 2006). This model has contributed to elucidate the roles of cytokines, growth factors and signalling pathways involved in pulmonary fibrosis. It has helped to determine TGF-β as one of the key factors in the development of pulmonary fibrosis (Zhao J et al, 2002). A number of novel drugs interfering with TGF-β have been investigated in the bleomycin model; one of them is decorin, an endogenous proteoglycan and known TGF-β1 inhibitor. It has been shown that intratracheal administration of decorin using an adenovirus vector leads to substantial reduction of the fibrotic response to bleomycin (Kolb M et al, 2001).

In vitro studies on isolated fibrocytes contribute to elucidate the role of these cells in pulmonary fibrosis. Fibrocytes express a number of cell markers including chemokine receptors and adhesion molecules. They partecipate in tissue remodelling by producing ECM proteins and by secreting MMPs. Fibrocytes are an important cellular source of inflammatory cytokines, chemokines, and growth factors that contribute to important autocrine and paracrine signals within the tissue microenvironment. These cells can differentiate into other mesenchymal cells, such as myofibroblasts and adipocytes (Strieter RM et al, 2007).

- **Systemic sclerosis (SSc)**

SSc is a clinically heterogeneous generalized disorder affecting the connective tissue of the skin and internal organs such as gastrointestinal tract, lungs, heart and kidneys. The hallmarks of SSc are autoimmunity and inflammation, widespread vasculopathy affecting multilpe vascular beds, and progressive interstitial and perivascular fibrosis. Patients with

SSc are commonly classified into two distinct subsets on the basis of the pattern of skin involment. Diffuse cutaneous SSc is dominated by rapidly progressive fibrosis of the skin, lungs, and other internal organs. By contrast, limited cutaneous SSc is dominated by vascular manifestations, and skin and organ fibrosis is generally limited and slow to progress. Although clinical outcomes have improved considerably, SSc is still considered incurable, and the diffuse cutaneous form carries the highest risk of fatality among connective tissue diseases, with 55% survival at 10 years (Mayes MD et at, 2003). Fibrosis is the most characteristic pathological hallmark of SSc, and it is especially prominent in the diffuse cutaneous form of the disease. Progressive replacement of tissue architecture by collagen-rich ECM results in functional impairment of affected organs. The fibrotic process is most prominent in the skin, lungs, gastrointestinal tract, heart, tendons and ligaments, and endocrine glands; widespread perivascular fibrosis also occurs. Fibrotic damage to these affected organs accounts for much of the morbidity and mortality associated with SSc. Fibroblasts explanted from lesional skin or the fibrotic lungs of patients with SSc displayed an abnormal activated phenotype that persisted for several passages in vitro (LeRoy EC, 1974). Persistent fibroblast activation in the absence of the fibrotic tissue milieu, confirmed very recently by DNA microarray studies (Gardner H et al, 2006) indicates autonomous, signal-indipendent alterations in cell function. The SSc phenotype is characterized by enhanced ECM synthesis, constitutive secretion of cytokines and chemokines, and increased expression of cell surface receptors for fibrogenic signalling mediators (Pannu J and Trojanowska M, 2004). SSc dermal fibroblasts can be readily isolated

and cultured and retain their ability to overexpress type I collagen and connective tissue growth factor. Thus examination of the phenotypic- and molecular-difference among normal fibroblasts from nonlesional and lesional areas of SSc patients should yield valuable insight into the molecular nature of scar tissue formation and progression in chronic fibrotic disease in general.

- **Liver fibrosis**

Liver fibrosis represents the final common pathway of virtually all chronic liver diseases. Advanced fibrosis is charachterized by an accumulation of ECM rich in fibrillar collagens (predominantly collagen I and collagen III) and is associated with an increased risk of liver cancer. As in other tissues, the fibrotic component of the liver's wound-healing response is mediated by myofibroblasts. In the injured liver, the myofibroblast is potentially derived from a number of cellular sources, foremost of which is the hepatic stellate cell (HSC) (Geerts A, 2001).

Several experimental animal models of liver disease support the role of the HSC as the primary cell responsible for excess collagen production during fibrogenesis. The localization of collagen proteins and increased mRNA levels within HSCs isolated from experimental models of liver fibrosis has been demonstrated (Milani S et al, 1990). Moreover, HSCs isolated from animal models of liver fibrosis synthesize more collagen than those from control animals (Schuppan D, 1992). However, although the HSC has historically been considered the primary hepatic cell type responsible for excess deposition of ECM during liver fibrosis, evidence mounting that other cell types also provide contribution. These additional fibrogenic cells include myofibroblasts, interstitial fibroblasts, and bile duct epithelial cells (Herbst H et al, 1997).

- **Cardiovascular fibrosis**

Fibrosis appears to be an integral component of most cardiac pathologies. The increased presence of ECM proteins within the myocardium results in an alteration of ventricular properties that causes both systolic and diastolic dysfunction. This environment impedes anisotropic or linear conduction leading to the development of arrhythmia. Similarly, excessive production of extracellular proteins within heart valves results in leaflet thickening and impaired motion with associated valvular dysfunction. In the heart the developing of fibrosis is prevalently due to TGF-β1. TGF-β1 is primarly secreted by cardiac fibroblasts. In turn TGF-β1 induces the differentiation of cardiac fibroblasts to more active myofibroblasts. Dilated, ischaemic and hypertrophic cardiomyopathiess are all associated with raised levels of TGF-β1. In fact, the pathogenic effects of TGF-β1 have now been suggested to play a major role in valvular disease and arrhytmia, particularly atrial fibrillation.

TGF-β1 is also involved in raising the production of cellular adhesion molecules, which are thought to increase myofibroblast survival and activity. The TGF-β1-Smad pathway appears to be an integral component of fibrosis within the heart, however, the pathway and its regulation have yet to be fully elucidated.

After infarction, the myocardium undergoes a reparative process where necrotic cardiac tissue is replaced by ECM proteins, in an effort to protect the integrity of the heart wall. This “scarred tissue” is dynamic, constantly producing and resorbing collagen. Functionally, the remainder of the heart makes up for the lost muscle tissue by increasing levels of fibrosis and inducing myocite hypertrophy, allowing for initial compensation of ventricular function (Khan R and Sheppard R, 2006).

- **Progressive kidney disease**

  Renal fibrosis is regarded as the final common pathway for most forms of progressive renal disease, and involves glomerular sclerosis and/or interstitial fibrosis. The model of unilateral ureteral obstruction (UUO) in rodent is the principal animal model for studying renal fibrosis. UUO was shown to result in proliferation of renal interstitial fibroblasts and their transformation into myofibroblasts. Subsequent studies showed increased interstistial collagens I, III and IV, fibronectin, and heparin sulfate proteoglycan (Sharma AK et al, 1993).

  One mechanism concerns the basic belief that the occasional interstitial fibroblast present in the normal kidney starts dividing due to the production of angiotensin II, TGF-β1, and other growth factors by injured renal epithelial cells. Another mechanism is based on the idea of EMT (see 1.5 Myofibroblast origin).

  This idea is rooted in the observation that renal epithelial cells dedifferentiate to a degree during various forms of renal disease. These dedifferentiated cells can then penetrate the basement membrane and redifferentiate into a myofibroblastic cell expressing α-SMA and collagen I. Recent studies have revealed major pathways leading to the development of renal interstitial fibrosis: interstitial infiltration by macrophages, which produce cytokines responsible for tubular apoptosis and fibroblast proliferation and activation; tubular cell death by apoptosis and necrosis leading to the formation of atubular glomeruli and tubular atrophy; phenotypic transition of resident renal cells. In progressive kidney disease, endothelial cells and pericytes are the major source of renal collagen-producing cells.

The role of TGF-β1

Of the myriad of cytokines that are involved in wound healing, TGF-β1 has the broadest effects. It is widely accepted that TGF-β1 and its downstream Smad signalling play an essential role. Through intensive investigations over the last decade, much has been learned about the importance of TGF-β1 in tissue fibrosis.

TGF-β1 is a member of a family of polypeptide growth factors that play pivotal roles in development and tissue homeostasis.

Members include: activins and inhibins, bone morphogenetic proteins (BMPs), growth and differentiation factors, Mullerian inhibitory substance, glial cell line-derived neurotropic factor (GCNF), and TGF-β (Kingsley DM, 1994). TGF-β family members are dimeric molecules comprised of two monomers, a β–strand and an α-helix.

1. **TGF-β pathway**

TGF-β signalling is initiated by ligand binding to a membrane-associated receptor complex that has serine/threonine kinase activity. This receptor complex phosphorylates specific Smad proteins, which then transduce the ligand-activated signal to the nucleus. Smad complexes regulate target gene transcription either by directly binding DNA sequences, or by complexing with other transcription factors or co-activators. There is extensive crosstalk between the TGF-β signaling pathway and other signalling systems, including the mitogen-activated protein kinase pathways (Cheng J and Grande JP, 2002).

Once activated, TGF-βs bind to a heteromeric receptor complex consisting of one TGF-β type I and one TGF-β type II receptor (Roberts AB, 1999). Both of these receptors possess tyrosine kinase activity. TGF-β may also be presented to the type II receptor by the accessory proteins

betaglycan and endoglin (Guerrero-Esteo M et al, 2002), which may modify the cellular response to TGF-β (Eickelberg O et al, 2002). After binding to TGF-β receptors, TGF-β signals within the cell through the Smad family of transcriptional activators.

In the presence of TGF-β ligand, the receptor-activated Smads (R-Smads), Smad2 and 3, are phosphorylated directly by the TGF-β receptor I kinase, bind to the commom mediator Smad, Smad4, and translocate into the nucleus. TGF-β induction of gene expression often requires the transcriptional cofactor p300, the TGF-β-mediated phosphorylation of Smad3 potentiates the association between Smad3 and p300 (Shen X et al, 1998). Smad2, on the other hand, is not believed to bind DNA directly, but rather requires a nuclear DNA binding protein of the family Fast (Fast-1) to bind DNA, in association with Smad4, and to activate transcription in response to TGF-β and activin (Liu B et al, 1999). A third group of Smad proteins, the inhibitory Smad (I-Smad), such as Smad6 or Smad7, prevents R-Smad phosphorylation and subsequent nuclear translocation of R-Smad/Smad4 heterocomplexes; it appears that Smad7 competes for binding for Smad2 and Smad3 to the TGF-βR1. TGF-β also induces Smad7 through a Smad3- and 4-dependent mechanism, suggesting that TGF-β can suppress its action via the induction of Smad7 (Von Gersdorff G et al, 2000) (Fig. 23).

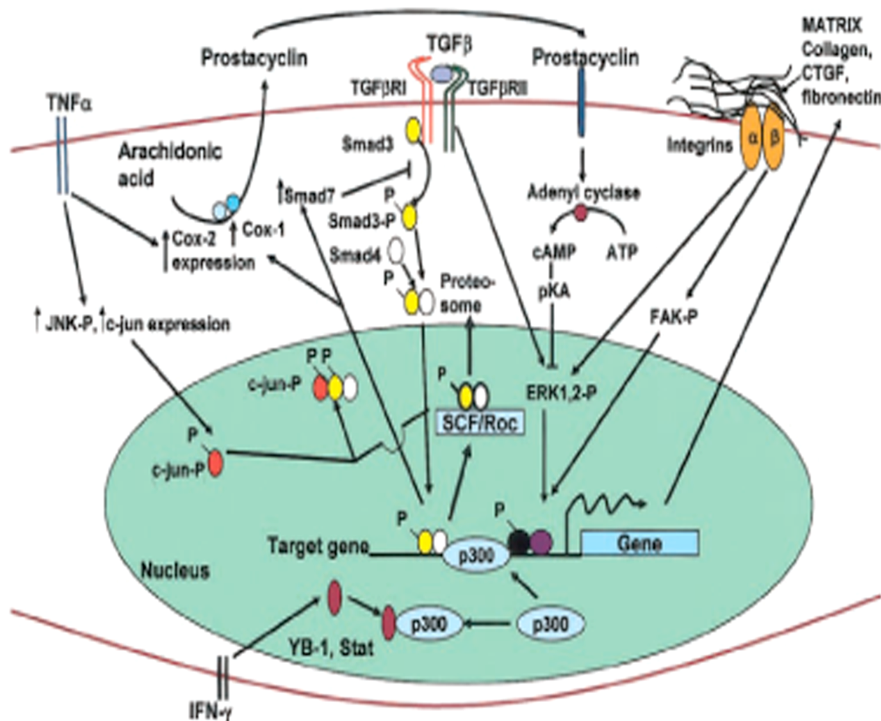


Fig. 23. Model showing positive and negative controls on TGF-β signalling that can occur after immediate-early signalling events. Smad-dependent activation of profibrotic genes is controlled by several methods. TGF-β terminates the induction of its own target genes by the induction of Smad7 and prostacyclin. Induction of CTGF and ED-A fibronectin enhances the profibrotic effects of TGF-β. TNF-α suppresses TGF-β–dependent transcriptional responses by increasing prostacyclin synthesis, elevating c-jun levels, and inducing JNK. INF-g suppresses the action of the Smad coactivator p300 by inducing YB-1 and Stat, which sequester free p300. Prostacylcin suppresses induction of profibrotic genes by suppressing MEK/ERK.

Recent studies have underscored the importance of TGF-β1 in regulation of cell proliferation and ECM synthesis and deposition. TGF-β1 is a key mediator of fibrogenesis in various tissues by virtue of its ability to increase the synthesis of ECM proteins and regulate the autocrine

synthesis of PDGF and connective tissue growth factor (CTGF) while it is also able to decrease the synthesis of MMPs by increasing the synthesis of TIMPs.

2. TGF-β1 induction of ECM genes

TGF-β1 causes matrix deposition in mesenchymal cells in culture by promoting expression of ECM genes and suppressing the activity of genes such as MMPs, which degrade ECM. TGF-β1 increases the synthesis of many ECM proteins, including collagen types I, II, III, IV, V and VII, fibronectin, thrombospondin, osteopontin, tenascin, elastin and decorin. TGF-β1 decreases production of proteases that break down ECM macromolecules, including serine thio, and MMPs, plasminogen activator, stromelysin, and collagenase. TGF-β1 increases the synthesis of inhibitors of metalloproteinases such as plasminogen activator inhibitor-1 (PAI-1) and TIMPs. Expression of integrins, cell surface receptors for ECM, is induced by TGF-β1. Integrins facilitate the attachment of cells to specific matrix proteins (Cheng J and Grande JP, 2002).

3. TGF-β1 and myofibroblastic differentiation

Under pathological conditions, TGF-β1 coordinate a cross talk between parenchymal, inflammatory, and collagen-expressing cells and plays a key role in stimulating fibrosis. Overexpression of TGF-β1 in transgenic mice results in fibrosis in multiple organs, suggesting that TGF-β1 is a major pro-fibrogenic cytokine (Bataller R, and Brenner DA, 2005).

TGF-β1 is known to play a role in fibrosis in DMD. It is highly expressed in DMD muscle (Bernasconi P et al, 1995), DMD myotubes (Zanotti S et al, 2007) and in DMD muscle-derived fibroblasts (Zanotti S et al, 2009). TGF-β1 is also a major player in connective tissue cell

proliferation (Bernasconi P et al, 1999; Zanotti S et al, 2009). TGF-β1 is the most accepted stimulator of myofibroblastic differentiation.

4. Myofibroblast

The key cellular mediator of fibrosis is the myofibroblast, which when activated serves as the primary collagen-producing cell.

The myofibroblast was initially identified by means of electron microscopy in granulation tissue of healing wounds as a modulated fibroblast, exhibiting features of smooth muscle (SM) cells, such as bundles of microfilaments, with dense bodies scattered in between, and gap junctions (Gabbiani G et al, 2001). The presence of myofibroblasts has successively been described in practically all fibrotic situations characterized by tissue retraction and remodelling.

5. Myofibroblast origin

A growing body of evidence from studies conducted in different organs suggests that resident fibroblasts are the primary source of ECM in the course of fibrosis. Resident myofibroblats arise from a population of tissue specific fibroblasts that proliferate and undergo activation in response of injury (Kumar V et al, 2005).

Classical myofibroblats differentiate from a mesenchymal lineage and, therefore, lack expression of lymphoid markers such as CD45 or CD34. In presence of fibrogenic stimuli in culture, such as TGF-β1 or certain types of ECM (fibronectin, collagen type I), they rapidly acquire a myofibroblastic phenotype, including expression of α-SMA, secretion of collagen type I and III, and contractility. Resident fibroblasts/myofibroblasts are believed to be the major source of collagen producing cells in lungs, skin, kidneys, and liver. In fibrotic lungs, myofibroblasts arise from pre-existing fibroblasts such as peribronchiolar

and perivascular adventitial fibroblasts (Lama VN and Phan SH, 2006). Dermal fibroblasts give rise to a population of myofibroblasts and induce scarring of injured skin (Desmouliere A, et al, 2005). In diseased kidneys, activation of cortical fibroblasts into myofibroblasts correlates with development of tubolointerstitial fibrosis (Remuzzi G and Bertani T, 1998). In fibrotic livers, the primary collagen producing cells originate in the liver but possess unique features. The majority of liver myofibroblats arises not from fibroblasts, but from HSCs. In response to injury, quiescent HSCs undergo morphological and functional changes and activate into myofibroblasts and are capable of phagocytosis and antigen presentation (Bataller R et al, 2003).

It is now widely believed that myofibroblasts can also derive from other tissue resident cell types also contribute to fibrosis (Hinz B et al, 2007). Pericytes and vascular smooth muscle cells (SMC) are an additional source of local myofibroblasts in scleroderma, liver, and glomerular fibrosis (Desmouliere A et al, 2003). In renal fibrosis, mesangial cells (kidney perycite) undergo activation (Harris RC and Neilson EG, 2006).

Bucala et al have identified a unique circulating fibroblast-like cell that is derived from bone marrow stem cells. These blood-bone mesenchymal progenitors have a fibroblast-and/or myofibroblast-like phenotype and were termed fibrocytes (Bucala R et al, 1994). Fibrocytes are implicated in fibrosis in the skin, lungs, kidneys, and liver. Fibrocytes have dual characteristics of lymphoid cells and fibroblasts and are defined as collagen producing $CD45^+$ cells. They also secrete growth factors and cytokines, which promote deposition of ECM in the local area of fibrosis (Quan TE et al, 2006).

In addition to resident mesenchymal fibroblasts, myofibroblasts can be derived from epithelial cells via EMT (Kalluri R and Neilson EG, 2003). EMT is “a process when fully differentiated epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells (Zavadil J and Bottinger EP, 2005). Several steps are critical for EMT: 1) disruption of local basement membrane; 2) loss of epithelial cell adhesion; 3) reprogramming of signalling machinery and de novo synthesis of α-SMA; and 4) rearrangement of cytoskeletal proteins and transmigration of epithelial cell through the basement membrane into interstitial space (Kalluri R and Neilson EG, 2003) (Fig. 24).

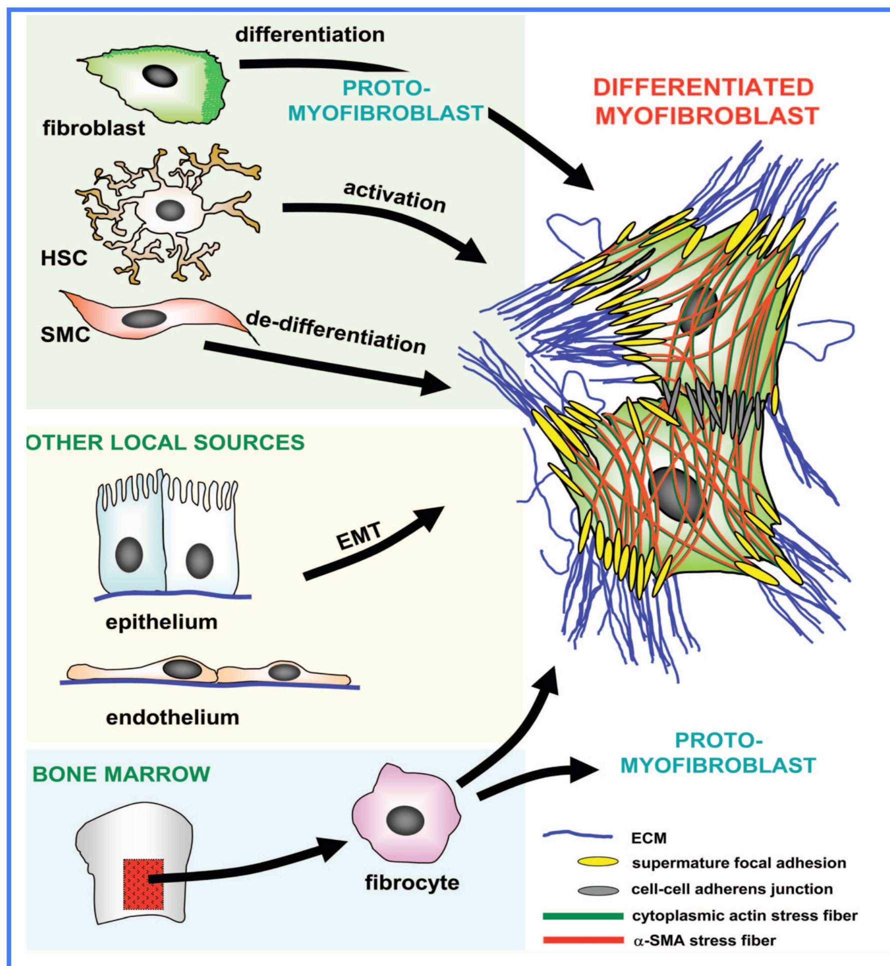


Fig. 24. Differentiated myofibroblasts are characterized by increased production of ECM proteins and by the development of α-SMA-posititve stress fibers that are connected with the ECM at site of supermature FAs and between cells via adherens junctions. The main myofibroblast progenitor after injuty of different tissues seems to be locally residing fibroblast, which transiently differentiates into a protomyofibroblasts, characterized by α-SMA-negative stress fibers.

6. From fibroblasts to myofibroblasts

It is accepted that the myofibroblastic modulation of fibroblastic cells begins with the appearance of the protomyofibroblast, whose stress fibers contain only β- and γ-cytoplasmic actins and evolves, but not necessarily always, into the appearance of the differentiated myofibroblast, the most common variant of this cell, with stress fibers containing α-SMA (Darby I et al, 1990).

Myofibroblasts can, according to the experimental or clinical situation, express other SM cell contractile proteins, such as SM-myosin heavy chains or desmin; however, the presence of α-SMA represents the most reliable marker of the myofibroblastic phenotype.

Although the modulation toward the protomyofibroblast is at present not well explored, the switch from the protomyofibroblast to the differentiated myofibroblast has been related to the production by inflammatory cells, and possibly by fibroblastic cells, of TGF-β1. The action of TGF-β1 depends on the local presence of the cellular fibronectin splice variant ED-A (Fig. 25).

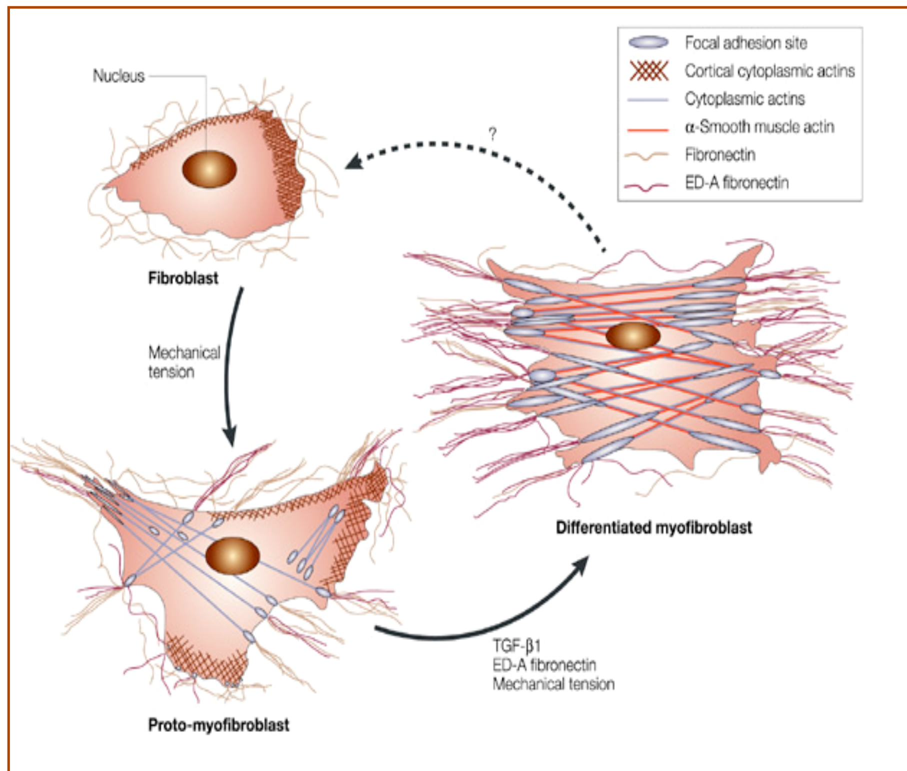


Fig. 25. The schematic drawing of myofibroblastic modulation from fibroblastic cells.

The contractile units of myofibroblasts contain bundles of actin with non-muscle myosin (Dugina V et al, 2001). Intracellular actin fibers link to extracellular fibronectin via transmembrane integrins (Dugina V et al, 2001), and thereby provide a mechano-transduction system that transmits forces generated by the stress fibers to the ECM (Burridge K and Chrzanowska-Wodnicka M, 1996). In addition, extracellular mechanical signals can become intracellular signals by reversal of the direction of force transmission (Burridge K and Chrzanowska-Wodnicka M, 1996).

Myofibroblasts are present in abundance within fibrotic lesions and thus contribute to the excessive scarring observed in lesions of fibrotic disease (Gabbiani G, 2003).

7. The role of ECM in activation of myofibroblasts

In addition of TGF-β1 and other fibrogenic stimuli, fibrotic changes in the cellular microenviroment can significantly affect the phenotype of tissue-resident collagen producing fibroblasts (Hinz B et al, 2007). Adjacent connective tissue serves as a structural scaffold that determines fibroblat morphology. Depending on the ECM composition, fibroblasts maintain quiescence or activate into myofibroblats. In the presence of laminin, a component of the basement membranes, fibroblasts remain quiescent. Fibroblasts subjected to extracellular stress caused by abnormal ECM (fibronectin, collagen type I and III), proliferate and obtain a myofibroblastic phenotype (Kisseleva T and Brenner DA, 2008). Tension, rigidity, or stiffness of ECM is another characteristic that regulates fibroblast phenotype (Eckes B et al, 2006).

Ultrastructural analysis of myofibroblasts in fibrotic and wound granulation tissue has revealed massive cell-ECM contacts that are not formed by normal connective tissue fibroblasts. Mechanically stressed fibroblasts exhibit a phenotype characteristic of scleroderma fibroblasts (deposition of ECM and synthesis of fibrotic cytokines), while fibroblasts on relaxed ECM secrete proteases and non-fibrogenic cytokine (Eckes B et al, 2006).

8. Myofibroblast contraction and fibrosis

The role of the fibroblast in determining organ shape during embryonic development is presently more and more accepted (Doljanski F, 2004). The most plausible mechanism of this morphogenetic action is ECM shape remodelling, which in turn influences epithelial architecture. The correct repair of connective tissue in a given organ requires the proper reconstitution of its support function and an appropriate tensile strength must be recreated. α-SMA-expressing myofibroblasts not only promote contraction but also synthesize elevated levels of both ECM components and matrix degrading proteases (Desmouliere A, et al 2005).

Myofibroblats are responsible for matrix reorganization as a result of changes in contractile forces (Grinnell F, 1994), thereby remodelling tissue and matrix and promoting wound closure. Once the wound is healed, myofibroblasts become apoptotic and are removed by phagocytosis. However, if myofibroblasts do not undergo apoptosis and continue to remodel the ECM, the connective tissue becomes increasingly strong. The persistence of myofibroblast within a fibrotic lesion leads to excessive scarring with the functional impairment of the affected organ. In muscle, this is demonstrated as contractures, which shorten the whole muscle and tighten it between attachments. The interaction between the myofibrolasts and its surrounding ECM play an important role in the resultant mechanical properties of the connective tissue (Hinz B and Gabbiani G, 2003).

MYOSTATIN

Myostatin is a highly conserved member of the TGFβ superfamily and possesses all of structural components common to the family: nine invariant cytseine residues, a furin-type proteolytic processing site, and a bioactive C-terminal domain (Lee SJ, 2004). Its expression in mammals is limited primarily to skeletal muscle, which in mammals appears to be the principal target tissue. The muscle mass of myostatin-null cattle, sheep, mice, and humans is dramatically increased and produces a phenotype often referred to as “double muscling”. Enhanced muscle growth in these animals is due to increases in both cell number and cell size, which results in larger and heavier myofibers. Decorin binds myostatin at a 1:1 ratio and with relatively high affinity, and prevents receptor activation (Miura T et al, 2006). The spatiotemporal expression patterns of both myostatin and decorin in rat skeletal muscle are similar and are consistent with decorin’s ability to sequester myostatin outside the cell (Nishimura T et al, 2007). Myostatin and TGF-β1 contribute to maintain fibrosis in some forms of muscular dystrophy because both factors induce myogenic cells to differentiate into myofibroblasts (Li Y et al, 2004). Myostatin also stimulates fibroblast proliferation in vitro as well as the secretion of TGF-β1 from mouse C2C12 myoblasts (Zhu J et al, 2007).

Fibrosis in DMD muscle

The changes that occur in dystrophin-deficient muscle are complex, and unpicking the casual relationships between them is not straightforward. Important abnormalities of dystrophin-deficient muscle cells have been demonstrated in three areas: calcium homeostasis, an increased susceptibility to mechanical damage, and increased membrane permeability. Calcium homeostasis is critical to many aspects of muscle function. Hypercontracted fibers are the earliest morphological abnormality of DMD and were ascribed to persistently raised intracellular [Ca^{+2}], DMD muscle biopsies showed an increased in the number of fiber positive for a histochemical calcium stain.

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei. Minor lesions inflicted by day-to-day deterioration elicit only a slow turnover of its constituent multinucleated muscle fibers. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage.

In DMD muscles there is constant cycling of degeneration and regeneration, but with age the repair phase becomes continually less successfull. Muscles become progressively weaker and are replaced with fat and connective tissue. Interestingly, the decline in regenerative capacity is not from a morphological difference in satellite cells or any reduction in satellite cell numbers (Watkins SC et al, 1988). However, satellite cell lifespan may be shorter in DMD because the telomeres shorten from the repetitive regeneration. This limits the number of cell cycles that one cell can undergo (Decary et al, 2000). In DMD muscle, nitric oxide synthase-1 is downregulated, causing an inhibition of nitric

oxide release, so satellite cells are over-activated (Anderson JE, 2000). Early in DMD, satellite cells easily become activated. Activated satellite cells that migrate into the inter-fiber space are called myoblasts. Muscle hypertrophy is an early consequence of muscle weakness and this is partly enabled by satellite hyperactivity. Ongoing repair produces an increasing value for the central nucleation index, as repaired fiber segments have central nuclei. However, the central nucleation index can decrease as fibers are lost and replaced with fat and connective tissue during the later progression of dystrophy. As regeneration continues and myoblast divisions accumulate, there will be fewer satellite cells able to enter or complete the cell cycle; therefore muscles become progressively weaker and fibers are lost.

In DMD, fibrosis is demonstrated by large increases in type I and type III collagens in the muscles (Finsterer J et al, 2006) and of collagen type VI (Zanotti S et al, 2005) (Fig. 26).

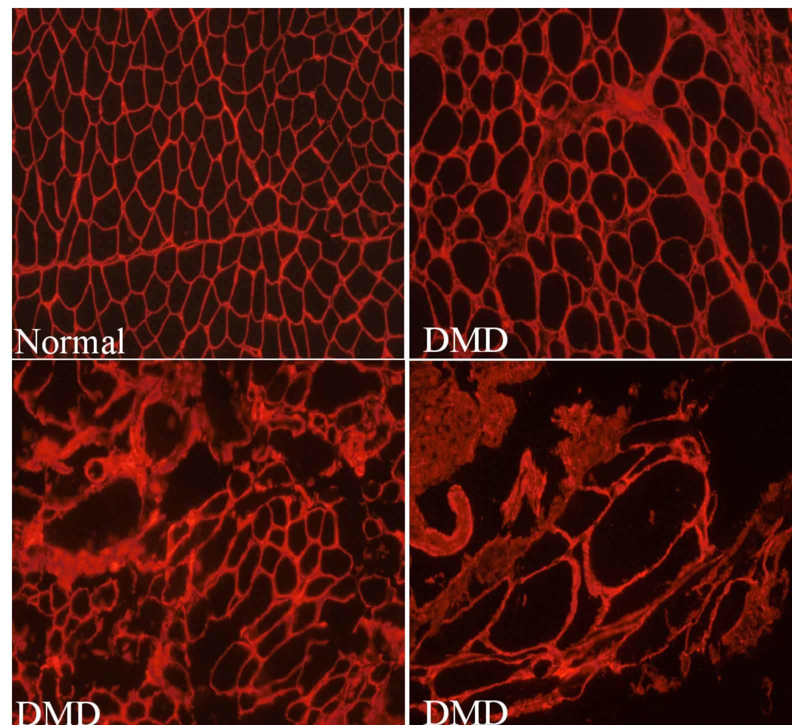


Fig. 26. Representative photomicrographs showing immunolocalization of collagen VI in sections of muscle from control and three DMD patients

Fibrosis is correlated with muscle damage and thought to play a crucial role in the mechanisms that lead to death. Fibrosis builds up as the muscle tissue is damaged, and accumulates via inflammatory and regenerative processes. Fibrosis and loss of muscle function together lead to respiratory and heart failure (due to DMD effects on respiratory and cardiac muscles) as well as to the decline in limb muscle function and contractile strength (Fig.27).

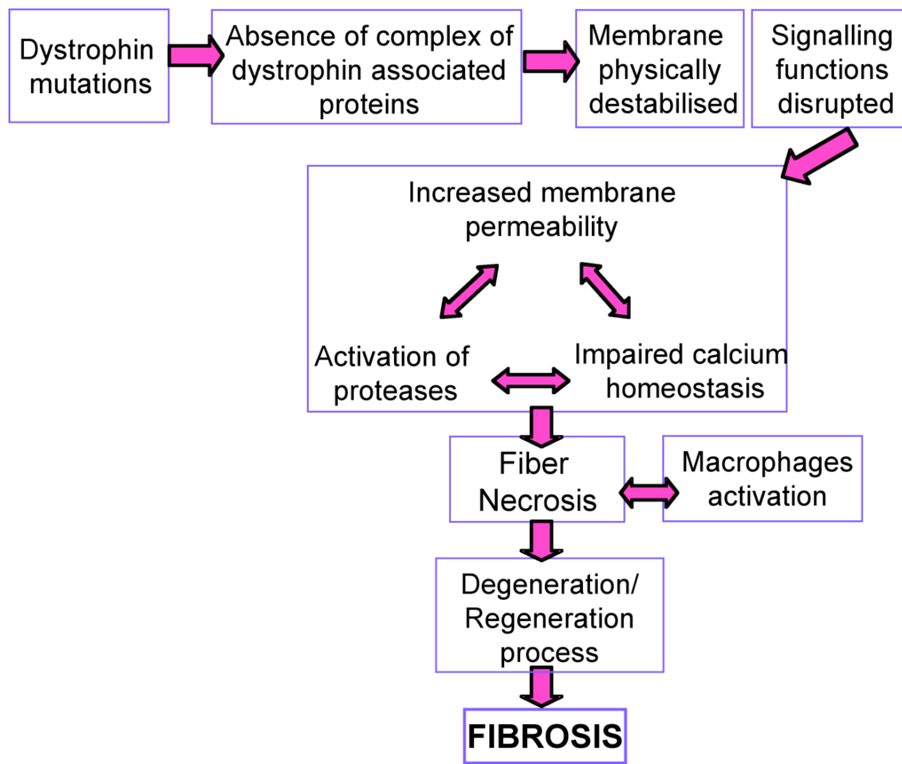


Fig. 27. Schematic rapresentation of mechanisms leading to fibrosis in DMD muscle.

Cardiomyopathy in DMD is characterized by large amounts of fibrosis of the myocardium, especially in the left ventricle. This is most likely due to increased myofibroblast activity. Fibrosis impairs muscle function and causes weakness. Often fibrosis is progressive and can contribute to the patient's inability to carry out ordinary tasks of independent living, such as grasping objects or walking.

Chapter 2

Scope of the thesis

The principal goal of this study is to increase understanding of pro-fibrotic mechanisms in dystrophic muscle by reconstructing, in an *in vitro* muscle cell-fibroblast culture model, the various steps of ECM deposition. Fibrosis is a complex mechanism not completely understood, triggered for repairing the injured tissue and for isolating it from the healthy surroundings.

We expect that our *in vitro* studies will help to better understand which are the major players in muscle fibrosis. Furthermore, our studies will provide clues to the development of anti-fibrotic therapies that, in the absence of specific treatments, may improve muscle function and slow disease progression in DMD and other muscular dystrophies.

Chapter 2

We performed this study in order to investigate whether ECM components contribute to the severe fibrosis observed in DMD skeletal muscle. We analyzed several ECM components as transcripts and proteins in primary DMD and control myotube culture. In particular we evaluated TGF-β1, myostatin, decorin, MMP-2, MMP-9, TIMP-1, -2, -3, collagens I and VI. We have found that transcript expression and protein modulation of several ECM components is altered in DMD muscle cells in vitro indicating their contribute to the pathological process.

Chapter 3

To probe profibrotic mechanisms in DMD muscle we isolated fibroblasts from DMD and control biopsies and induced transdifferentiation to myofibroblasts by TGF-β1 treatment. The study has revealed several differences between fibroblasts from DMD muscle and those from normal muscle, particularly as regards factors likely to affect ECM turnover. This study confirms that fibroblasts and myofibroblasts play a major role in muscle fibrosis and suggests that DMD fibroblasts have a profibrotic phenotype accentuated by TGF-β1 treatment.

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Chapter 3

Altered extracellular matrix transcript expression and protein modulation in primary Duchenne muscular dystrophy myotubes.

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ABSTRACT

Extent of muscle fibrosis contributes to disease severity in muscular dystrophies. To investigate whether extracellular matrix (ECM) components contribute to the severe fibrosis observed in Duchenne muscular dystrophy (DMD) skeletal muscle, we quantitated several ECM components (transcripts and proteins) in primary DMD and control myotube cultures. We evaluated the fibrogenic transforming growth factor-β1 (TGF-β1); the small pleiotropic proteoglycan decorin, involved in collagen fibrillogenesis and TGF-β1 modulation; metalloproteinases MMP-2 and MMP-9; tissue inhibitors of metalloproteinase (TIMP) 1, 2 and 3; collagens I and VI; and the tissue factor myostatin that inhibits muscle growth.

Dystrophic myotube cultures had significantly lower levels of decorin mRNA, as also observed in DMD muscle biopsies, and significantly higher levels of TGF-β1, myostatin, and collagens I and VI. MMP-2, TIMP-1 and TIMP-2 transcript levels were also significantly increased in DMD, but MMP-9 and TIMP-3 transcripts were unchanged. By zymography, MMP-2 activity was significantly higher in DMD than control. Protein levels were similar in DMD and controls but myostatin protein was significantly increased in DMD.

We have found that transcript expression and protein modulation of several ECM components is altered in DMD muscle cells in vitro, indicating that these cells contribute fundamentally to the pathological process, since the inflammation and degeneration characterizing DMD muscle in vivo are presumably absent in culture. Our findings that myostatin – potent inhibitor of satellite cell activation and muscle renewal – is increased, and that decorin – binder and downregulator of

TGF−β1 and myostatin – is decreased, may have implications for DMD therapy to reduce muscle fibrosis.

INTRODUCTION

Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene on the X chromosome, is one of the most common inherited human neuromuscular diseases, affecting 1 in 3500 male births. Dystrophin is crucial for maintaining the structural integrity of muscles and seems to do this by linking the internal cytoskeleton of muscle fibers to the extracellular matrix (ECM) via binding with the dystrophin associated glycoprotein complex (Blake et al., 2002). The main consequences of dystrophin absence are sarcolemma instability and increased muscle cell vulnerability to mechanical stress, resulting in fiber necrosis and fiber regeneration soon giving rise to progressive muscle wasting and replacement by connective tissue and adipose (fibrosis). Lack of dystrophin is also associated with a variety of complex secondary processes and with changes in the expression of many genes (Haslett et al., 2002).

Fibrosis, the most conspicuous pathological change in muscle, is a complex incompletely understood process characterized by excessive accumulation of collagen and other ECM components; it is regulated by mechanisms involving cell-cell and cell-matrix interactions, as well as by factors secreted into the ECM.

The ECM plays important roles in cell signaling and cell homeostasis (Raghow et al., 1994); it is composed of proteoglycans, fibrous proteins, adhesion molecules, and matrix-regulating enzymes. There

are several families of ECM-degrading enzymes, including the matrix metalloproteinases (MMPs). At least 24 MMPs are known in humans; they are secreted as zymogens and activated by proteolytic removal of a propeptide (Visse and Nagase, 2003). Two members of the MMP family, gelatinase A and B (MMP-2 and MMP-9, respectively), have been shown to degrade ECM components including denatured collagens, native type IV collagen, fibronectin, elastin and decorin, and seem to be involved in ECM remodeling (Senior et al., 1991; Kerrigan et al., 2000). Transcript and protein levels of these two enzymes are altered in several conditions characterized by fibrosis (Oggionni et al., 2006; Kossakowska et al., 1998; Rouet-Benzineb et al., 1999). MMP activity is regulated by transcriptional control, extracellular activation of zymogens, and tissue inhibitors of metalloproteinases (TIMPs) which form tight complexes with active enzymes (Maskos, 2005; Baker et al., 2002).

Abnormal connective tissue proliferation also occurs in hepatic fibrosis (Kossakowska et al., 1998), glomerulosclerosis (Schnaper et al., 1996), myocardial fibrosis (Li et al., 2000; Herpel et al., 2006), idiopathic pulmonary fibrosis (Selman et al., 2001) and biliary fibrosis (Milani et al., 1990). The extensive structural disorganization and remodeling that characterize the fibrotic process in these conditions involve imbalance of MMPs and TIMPs, as well as release of fibrogenic cytokines such as transforming growth factor-β1 (TGF-β1) (Mauviel, 2005).

Decorin, a component of the small leucine-rich proteoglycan family, sequesters TGF-β1 forming complexes that modulate the cytokine's biological activity (Yamaguchi et al., 1990; Noble et al., 1992;

Hildebrand et al., 1994) for this reason decorin has been used as antifibrotic agent in several models of tissue fibrosis (Border et al., 1992; Giri et al., 1997; Kolb et al., 2001; Logan et al., 1999; Fukushima et al., 2001). Recent studies have shown that decorin also binds myostatin (Miura et al., 2006), an inhibitor of muscle cell growth and member of the TGF-β superfamily. Thus the possibility arises that decorin could have a doubly beneficial action on muscle fibrosis: downregulation of profibrotic TGF-β1, and inhibition of the negative influence of myostatin on muscle growth.

In muscle tissue from DMD and *LAMA2*-mutated congenital muscular dystrophy (MDC1A) patients, TGF-β1 transcript levels have been shown to be greatly increased (Bernasconi et al., 1995; Zanotti et al., 2005) and decorin transcripts decreased (Zanotti et al., 2005). These changes are likely to be related to the ongoing inflammatory and degenerative-regenerative processes that characterize the muscle in these conditions (Burger and Dayer, 2002; McCroskery et al., 2005).

To further investigate TGF-β1, decorin and myostatin interactions in DMD fibrosis, we investigated transcript and protein levels in dystrophic and normal myotube cultures obtained from muscle biopsies of DMD patients and age-matched controls. We also assessed transcript levels of MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, and collagens I and VI. In addition, we used immunocytochemistry to evaluate the expression of MMP-2, MMP-9 and TIMP-2, and zymography to assess the enzyme activity of MMP-2.

EXPERIMENTAL PROCEDURES

Primary human skeletal muscle culture

Biopsies were taken from quadriceps of 7 DMD patients (aged 1-7 years) and 5 controls (aged 1-10 years) after informed parental consent. Investigations on human tissue were approved by our institutional review board. DMD was diagnosed by dystrophin testing and gene analysis. Primary myoblasts were derived directly from biopsied material by culturing in Dulbecco's modified Eagle's medium (DMEM) containing 20% heat-inactivated fetal bovine serum (FBS) (Cambrex Corporation, East Rutherford, NJ), 1% penicillin-streptomycin (Cambrex), L-glutamine (Cambrex), 10 mg/ml insulin (Sigma Aldrich, St. Louis, MO), 2.5 ng/ml basic fibroblast growth factor (bFGF) (Gibco Life Technologies, Carlsbad, CA), and 10 ng/ml epidermal growth factor (EGF) (Gibco). The medium was changed twice weekly and the cultures examined by inverted-phase microscopy: when they reached 70% confluence they were dissociated enzymatically with trypsin-EDTA (Sigma) and seeded for immediate propagation, or frozen in medium containing 10% DMSO (Sigma) for later propagation or other use.

To obtain myotubes, the myoblasts were seeded into 35 mm dishes at 2 x 10^4 cells/ml in DMEM proliferating medium. At 70% confluence, proliferating medium was changed to differentiating medium (DMEM, 1% penicillin-streptomycin, L-glutamine and insulin, without FBS or growth factors) and the myoblasts were allowed to differentiate to myotubes over 21 days.

Immunomagnetic myoblast enrichment

To obtain myoblast- enriched populations, primary myoblast cultures were sorted by immunomagnetic selection using the CD56 surface marker (Sinanan et al., 2004). 10^7 cells were mixed with 100 µl CD56-coated microbeads (Milteny Biotec, Bergisch Gladbach, Germany) and incubated at 4°C for 15 min. Unbound cells were removed by washing with PBS and subsequent centrifugation. The pellet containing CD56-positive myoblasts bound to microbeads was resuspended in PBS and loaded onto a midiMACS cell separator unit (Milteny Biotec). After allowing negative cells to pass through the column, CD56-positive cells were flushed out using the plunger and subsequently seeded onto collagen-coated Petri dishes and cultured as described above.

Myogenic capacity of cell cultures

The myogenic capacity of enriched myoblast cultures was assessed using an anti-desmin antibody (Dako, Copenhagen, Denmark). For each myoblast cell line desmin immunoreactivity was tested at seeding, at confluence, and after fusion. Four randomly-selected fields per dish were photographed and the desmin-positive cells counted and expressed as a proportion of total DAPI-stained nuclei.

We also assessed ability of myoblasts to fuse into myotubes (fusion index) as the mean percentage of nuclei in myotubes having three or more nuclei compared to the total number of nuclei (myoblasts + myotubes) found in 10 randomly selected areas per Petri dish 2 days after seeding (T0) and after 21 days in differentiating medium (T21). Samples were examined under a Zeiss Axioplan fluorescence

microscope.

cDNA synthesis

Total RNA was isolated from enriched myotube cultures after 21 days of differentiation using TRI Reagent (Ambion, Inc. Austin, TX) according to the manufacturer's instructions and checked spectrophotometrically for quantity and purity. One μg aliquots of RNA were reverse transcribed in the presence of 5X first strand buffer (Invitrogen), 1 mM of each deoxynucleoside triphosphate, 8 pM random hexamers, 10 μM dithiothreitol, 1 IU/μl RNAse inhibitor (Roche Molecular Biochemicals, Basel, Switzerland) and 10 IU/μl M-MLV reverse transcriptase (Invitrogen) with incubation at 37°C for 1 h and at 95°C for 5 min. The reaction product was stored at -20°C pending use. cDNA integrity was assessed by PCR amplification of human GAPDH (GenBank accession no. M33197) using specific primers (forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'). PCR conditions were: 94°C 1 min, 54°C 1 min, 72°C 1 min, for 35 cycles.

Synthesis of competitors

Homologous competitors were obtained by PCR as described (Zanotti et al., 2005) using specific primers designed to be amplified by the same primer as those amplifying the targets (decorin, TGF-β1, myostatin, MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, collagen I, collagen VI) yet distinguishable from them. The primers were designed with Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and were synthesized by NBS Biotech (Milan,

Italy). Primers and PCR conditions for competitor synthesis are shown in Table 1. The PCR bands containing the competitors were excised from the gels and purified using the NucleoSpin Extract 2-in-1 kit (Macherey-Nagel, Duren, Germany). Quality was checked electrophoretically and quantified spectrophotometrically; the competitors were sequenced to check identity on a 3100 Genetic Analyzer (Applied Biosystems, CA).

Target mRNA	GenBank accession	Forward primer (5'-3')	Reverse primer competitor (5'-3')	Target (bp)	Competitor (bp)	PCR condition
Decorin	NM_001920	TGAAGGCCACTATCATCCTCC	TCACCAAAGGTGTAAATGCTCCACTTTGTCCAGACC	387	245	94°C 1 min, 50°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 55°C 2 min, 72°C 1 min, 25 cycles
TGF-beta	NM_000660	CGACTCGCCAGAGTGGTTAT	GTCCTTGCGGAAGTCAATGTGCCATGAGAAGC	346	256	94°C 1 min, 51°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 56°C 2 min, 72°C 1 min, 25 cycles
Myostatin	NM_005259	CGATGCTGTCGTTACCCTCT	GAGGGGAAAACCTTCCATGTTAGCATATTAATTGGA	342	236	94°C 1 min, 53°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 57°C 2 min, 72°C 1 min, 25 cycles
MMP-2	NM_004530	TTGGCAGTGCAATACCTGAA	GGTCACATCGCTCCAGACTTGCGAGGGAAGAAGT	321	255	94°C 1 min, 51°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 56°C 2 min, 72°C 1 min, 25 cycles
MMP-9	NM_004994	GTGATTGACGACGCCTTTG	TCGAAGATGAAGGGGAAGTGCCGGGGCCAG	305	205	94°C 1 min, 53°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 57°C 2 min, 72°C 1 min, 25 cycles
TIMP-1	NM_003254	CTGTTGTTGCTGTGGCTGAT	TGCAGTTTTCCAGCAATGAGCCTAAGGCTT	305	205	94°C 1 min, 51°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 56°C 2 min, 72°C 1 min, 25 cycles
TIMP-2	NM_003255	CTGGACGTTGGAGGAAAGAA	GTCGAGAAACTCCTGCTTGGGTCCATCCAGA	345	244	94°C 1 min, 50°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 51°C 2 min, 72°C 1 min, 25 cycles
TIMP-3	NM_000362	CTGACAGGTCGCGTCTATGA	GGTCTGTGGCATTGATGAATGTTGGAGAGCATGT	322	223	94°C 1 min, 51°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 56°C 2 min, 72°C 1 min, 25 cycles
Collagen I(alpha2-COL1A2)	NM_000089	GACATGCTCAGCTTTGTGGA	CCTGTGGTCCAACAACTCCTACCAGGCTCA	490	386	94°C 1 min, 50°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 51°C 2 min, 72°C 1 min, 25 cycles
Collagen VI(alpha1-COL6A1)	NM_001848	CAGTGACGAGGTGGAGATCA	ATTCGAAGGAGCAGCACACTGCGCCGGTAC	476	374	94°C 1 min, 50°C 2 min, 72°C 1 min, 5 cycles 94°C 1 min, 51°C 2 min, 72°C 1 min, 25 cycles

Competitive PCR

PCR mixtures containing various known quantities (6 x10^4, 12.5 x10^4, 2.5 x 10^4, 5 x 10^5, 7.5 x 10^5, 1.25 x 10^6, 2.5 x 10^6, and 5 x 10^6) of competitor molecules were added to cDNA samples reversed transcribed from 0.04 µg of total RNA in 25 µl reaction volumes. Competitive PCR was carried out using the primers and melting temperatures (Tm) shown in Table 2. In all cases 35 cycles of 1 min at 94° C, 1 min at Tm, and 1 min of 72°C were used. The products were electrophoresed on 2% agarose gel. The DNA bands were visualized with 5 µg/ml ethidium bromide and photographed with a Kodak EDAS290 digital camera (Eastman Kodak, Scientific Imaging Systems, New Haven, CO). Fluorescence quantification of competitors and targets employed a Fluor-S MultiImager densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and Quantity One software version 4.2.3 (Bio-Rad). Logarithms of the fluorescence density of competitor and target were plotted against competitor copy numbers. The number of target RNA molecules was determined from the intersection of the regression curve on the x-axis (number of competitor copies). The results were expressed as number of copies of target gene mRNA/0.04 µg total RNA.

Target mRNA	GenBank accession	Forward primer (5'-3')	Reverse primer (5'-3')	Target (bp)	Competitor (bp)	Tm (°C)
Decorin	NM_001920	TGAAGGCCACTATCATCCTCC	TCACCAAAGGTGTAAATGCTCC	387	245	55
TGF-beta	NM_000660	CGACTCGCCAGAGTGGTTAT	GTCCTTGCGGAAGTCAATGT	346	256	57
Myostatin	NM_005259	CGATGCTGTCGTTACCCTCT	GAGGGGAAAACCTTCCATGT	342	236	56,2
MMP-2	NM_004530	TTGGCAGTGCAATACCTGAA	GGTCACATCGCTCCAGACTT	321	255	56
MMP-9	NM_004994	GTGATTGACGACGCCTTTG	TCGAAGATGAAGGGGAAGTG	305	205	56,2
TIMP-1	NM_003254	CTGTTGTTGCTGTGGCTGAT	TGCAGTTTTCCAGCAATGAG	305	205	55
TIMP-2	NM_003255	CTGGACGTTGGAGGAAAGAA	GTCGAGAAACTCCTGCTTGG	345	244	55
TIMP-3	NM_000362	CTGACAGGTCGCGTCTATGA	GGTCTGTGGCATTGATGAATG	322	223	56,4
Collagen I(alpha2-COL1A2)	NM_000089	GACATGCTCAGCTTTGTGGA	CCTGTGGTCCAACAACTCCT	490	386	56
Collagen VI(alpha1-COL6A1)	NM_001848	CAGTGACGAGGTGGAGATCA	ATTCGAAGGAGCAGCACACT	476	374	56

Protein extraction

Enriched myotube cultures were washed with PBS and lysed on ice in extraction buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet-P, 1% Triton-X 100) containing a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA) for 30 min. The lysate was centrifuged at 18000 *g* for 15 min at 4°C. The supernatant was collected and the protein concentration determined using the DC Protein Assay Reagent (Bio-Rad).

Zymography

For detection of MMP-2 and MMP-9 activity in the last change of differentiation medium at 21 days after the cells had been in it for 48 hours, medium first was concentrated 5x using Ultrafree-4 centrifugal

filter unit (30 kDa cut-off, Millipore Corporation, Billerica, MA, USA).
Ten µg of 5x concentrated medium samples were then diluted 1:1 in non-reducing buffer (0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, 0.1% bromophenol blue) and electrophoresed on 7.5% SDS-polyacrylamide gels containing 1 mg/ml gelatin type A from pig skin (Sigma). The gels were washed twice for 15 min in 2.5% (v/v) Triton X-100, rinsed with distilled H_2O at room temperature, and incubated overnight in developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 200 mM NaCl) at 37°C. The gels were then stained with 0.5% Coomassie brilliant blue R-250 (in 30% methanol, 10% acetic acid) for 3 hours and destained with aqueous methanol (30%) and acetic acid (10%). Areas where the gelatin had been degraded by proteolytic activity were seen as absence of staining, and were quantified densitometrically using a Fluor-S-Max Multi-Imager (Bio-Rad) and Quantity One software version 4.2.3 (Bio-Rad)

Western blot

Twenty-five µg aliquots of protein extract from myotube cultures at 21 days were solubilized in 2x Laemmli buffer (0.5 M Tris-Cl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 1% bromophenol blue), boiled, electrophoresed in 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Schleicher and Schuell Inc., Keene NH, USA). Membranes were probed with one of the following antibodies: mouse monoclonal anti-decorin (R&D Systems Inc, Minneapolis, MN, USA) dilution 1:250; rabbit polyclonal anti-MMP-2, anti-MMP-9, and anti-TIMP-2 (all from Biomol International, L.P.,

Plymouth Meeting, PA, USA), all diluted 1:250. Appropriate biotin-conjugated secondary antibody was then applied (1:2500 dilution; Jackson ImmunoResearch), followed by peroxidase-conjugated streptavidin (1:3000 dilution; Jackson ImmunoResearch), and detection with ECL chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

TIMP-2 secreted extracellularly was detected in the last change of differentiation medium at 21 days after the cells had been in it for 48 hours and after 5x concentration as described above. Ten µg samples of concentrated protein were solubilized in 2x Laemmli buffer, boiled, electrophoresed in 12.5% SDS-PAGE gel and transferred onto nitrocellulose membranes. As control in each gel 1.5 mg of recombinant TIMP-2 (Chemicon International Temecula, Ca, USA) was loaded.

Immunocytochemistry

After 21 days in differentiating medium on gelatinized glass coverslips, enriched myotube cultures were fixed in 4% paraformaldehyde, permeabilized in PBS 0.1% Triton-X 100 for 30 min. at 4°C, and incubated overnight with one of the following primary polyclonal antibodies: anti-decorin (gift from Dr. Larry Fisher, National Institute of Dental and Craniofacial Research, National Institute of Health, Bethesda, USA) (Fisher et al., 1995), anti-MMP-2, anti-MMP-9, anti-TIMP-2 (all from Biomol International), anti-TGF-β1 (BioSurce International, Camarillo, CA, USA) and anti-myostatin (Chemicon), all diluted 1:50. The cells were then incubated with biotinylated anti-rabbit IgG (1:250 Jackson

ImmunoResearch, Westgrove PA, USA) for 60 min; followed by rhodamine-avidin D (1:250 Vector Labs, Burlingame, CA) for 60 min, and finally with DAPI (Sigma) 1:3000 dilution for nuclear staining. As negative controls primary antibodies were omitted. All incubations were carried out at room temperature in a humid chamber.

For co-localization of MMP-2 and smooth muscle α-actin, MMP-2 or MMP-9 and emerin (Novocastra, Newcastle upon Tyne, UK) a mixture of the primary MMP-2 polyclonal antibody and the a-actin or emerin monoclonal antibody was used in the first incubation step, followed by biotinylated goat anti-rabbit IgG (Jackson), by rhodamine avidin D, and by Cy2-labelled goat anti-mouse IgG (Jackson). As control, cell cultures were incubated either with isotype-specific IgG, or with non immune rabbit IgG, or the primary antibody was omitted.

Myostatin positivity was quantitated in five randomly-selected fields per dish of three DMD and three control cultures by counting positive spots and expressed as a proportion of total DAPI-stained nuclei.

Immunostained preparations were examined either under a Zeiss Axioplan fluorescence microscope or a Bio-Rad confocal microscope.

Statistical Analysis

Results are expressed as means and standard deviations. Differences between the two groups (DMD and control) were assessed using the Wilcoxon non-parametric test and considered significant for $p \leq 0.01$.

RESULTS

Characterization and quantitation of cells

Growth was similar in dystrophic and control cell populations; in particular, after 21 days in differentiation medium total cell numbers were $9.11 \times 10^5 \pm 0.511 \times 10^5$ in control lines and $8.59 \times 10^5 \pm 0.023 \times 10^5$ in DMD cell lines. The fusion index was also similar after 21 days in differentiation medium: 5.146 % ± 1.45 for control and 6.38 % ± 0.96 for DMD (Fig. 1A). However the percentage of cells expressing the myogenic marker desmin was significantly higher ($p<0.01$) at seeding in DMD (17.45 % ± 3.5) than in control (4.96 % ± 2.09) cultures, but was similar at confluence (33.98 % ± 5.14 in DMD vs. 30.53 % ± 7.19 in controls; $p=0.238$) and after 21 days of differentiation (60.99 % ± 12 in DMD vs. 54.63% ± 17.22 in controls; $p=0.471$) (Fig. 1B). After 21 days of differentiation, both control and DMD cell populations consisted of a layer of myoblasts plus differentiated myotubes.

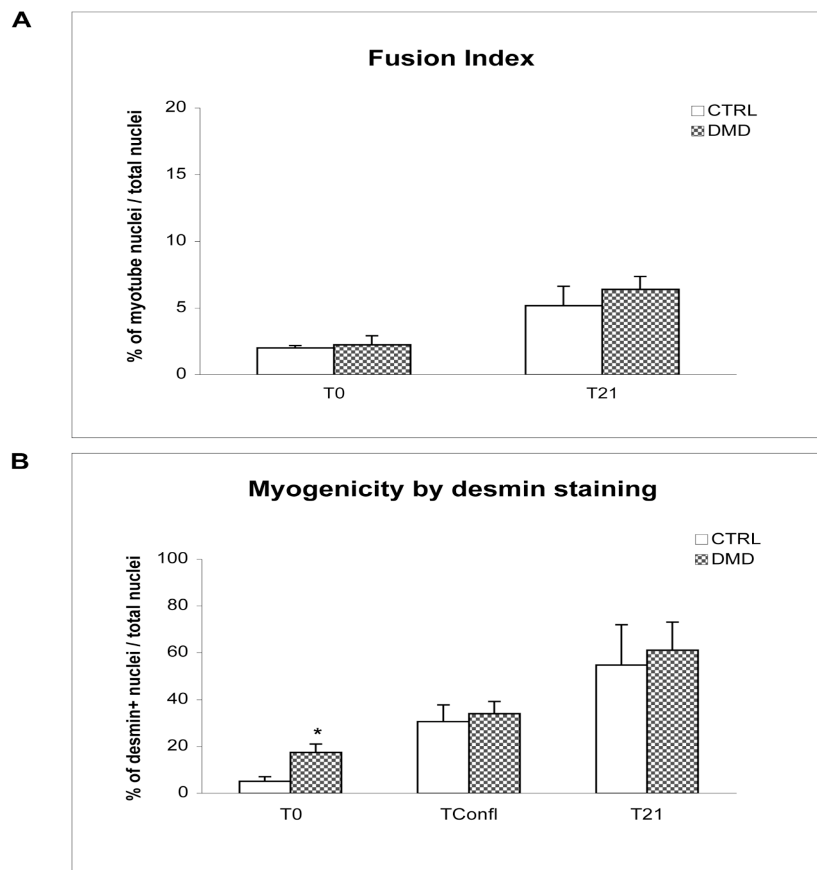


Figure 1. (A) Fusion indices determined 2 days after seeding (T0) and after 21 days of differentiation (T21) in DMD and control cell cultures. (B) Myogenicity of cell cultures at seeding (T0), at confluence (TConfl) and after 21 days of differentiation (T21), determined as the percentage of desmin-positive cells in each culture.

Competitive PCR

There were significantly fewer ($p<0.01$) copies of decorin mRNA in DMD than control cultures ($6.34 \times 10^5 \pm 0.1 \times 10^5$ vs. $9.53 \times 10^5 \pm 0.15 \times 10^5$). There were significantly more ($p<0.01$) copies of TGF-β1 transcript ($7.82 \times 10^5 \pm 0.3 \times 10^5$ vs. $5.08 \times 10^5 \pm 0.09 \times 10^5$) and also

significantly more ($p<0.01$) myostatin transcripts ($9.33 \times 10^5 \pm 0.87 \times 10^5$ vs. $3.86 \times 10^5 \pm 0.15 \times 10^5$) in DMD than control cultures (Fig. 2A).

Transcript levels of collagen I and collagen VI were significantly higher ($p<0.01$) in DMD than control cultures: collagen I was $7.23 \times 10^5 \pm 0.98 \times 10^5$ vs. $2.76 \times 10^5 \pm 0.49 \times 10^5$; collagen VI was $7.75 \times 10^5 \pm 1.7 \times 10^5$ vs. $4.46 \times 10^5 \pm 0.08 \times 10^5$ (Fig. 2B).

Transcript levels of MMP-2 were significantly higher ($p<0.01$) in DMD than controls ($9.32 \times 10^5 \pm 0.53 \times 10^5$ vs. $4.94 \times 10^5 \pm 0.12 \times 10^5$;), while MMP-9 mRNA levels did not differ significantly ($p=0.459$) between the groups ($7 \times 10^5 \pm 0.33 \times 10^5$ vs. $6.41 \times 10^5 \pm 0.12 \times 10^5$) (Fig. 2C).

Transcript levels of TIMP-1 and TIMP-2 were significantly higher ($p<0.01$) in DMD than control cultures: TIMP-1 levels were $1.13 \times 10^6 \pm 0.8 \times 10^5$ vs. $6.1 \times 10^5 \pm 0.1 \times 10^5$; TIMP-2 mRNA levels were $1.2 \times 10^6 \pm 1.01 \times 10^5$ vs. $6.8 \times 10^5 \pm 0.97 \times 10^5$. TIMP-3 mRNA levels did not differ significantly ($p= 0.09$) between DMD ($7.93 \times 10^5 \pm 0.81 \times 10^5$) and controls ($7.83 \times 10^5 \pm 0.62 \times 10^5$) (Fig. 2D)

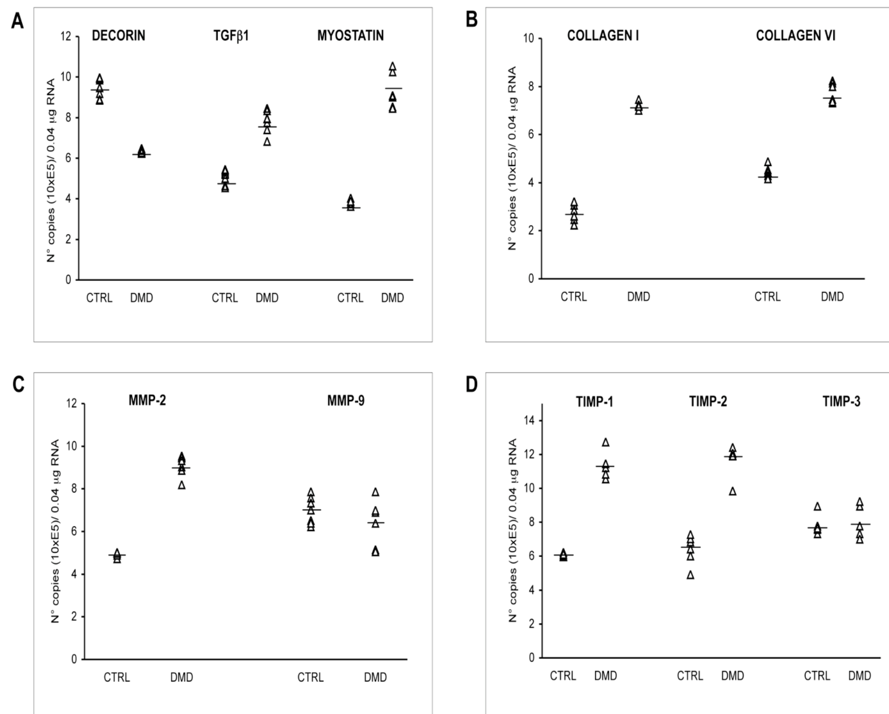


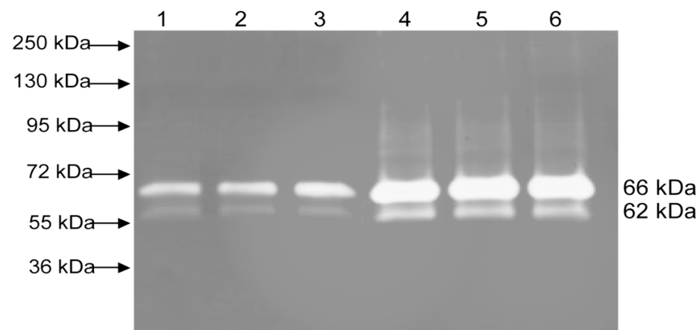
Figure 2. Competitive PCR data for (A) decorin, TGF-β1 and myostatin; (B) collagen I and collagen VI; (C) MMP-2 and MMP-9; and (D) TIMP-1, TIMP-2 and TIMP-3. Data are expressed as numbers of mRNA copies for each test in control and DMD cultures after 21 days in differentiation medium. Bars indicate mean values.

Zymography

Zymography showed the presence of bands of gelatinolysis at 66 and 62 kDa, corresponding to the inactive and active forms, respectively, of MMP-2 (Fig. 3A). Densitometric analysis showed that the 66 and 62 kDa bands from DMD cultures had significantly greater areas (indicating presence of more digested substrate) than bands from

control culture. For the 66 kDa band, areas were 2213.83 ± 138.0 mm^2 vs. 1041.66 ± 136.34 mm^2 ($p<0.01$) and for the 62 kDa band were 1009.83 ± 106.16 mm^2 vs. 562 ± 63.28 mm^2 ($p<0.01$) (Fig. 3B). The band at 95 kDa corresponding to MMP-9 was absent or barely discernible and could not be quantitated in media from either DMD or control cultures.

A



B

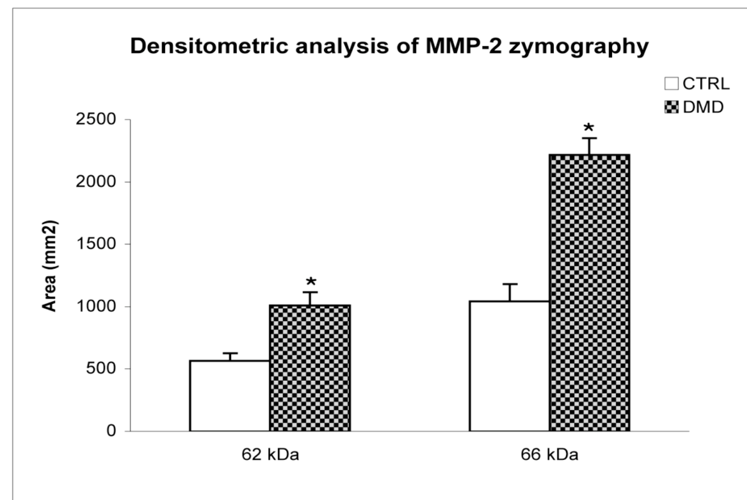


Figure 3. (A) Zymographic detection MMP-2 gelatinase activity in differentiated medium at 21 days from representative DMD (lanes 4-6) and control (lanes 1-3)

cultures. (B) Densitometric analysis of 66 and 62 kDa gelatinolytic bands of MMP-2. Asterisks indicate significant differences ($p<0.01$).

Western blot

The decorin, MMP-2, and MMP-9 bands from protein extracts were of comparable intensity by visual inspection in DMD and control, (Fig. 4A) while TIMP-2 bands were almost undetectable in both cases (not shown). TIMP-2 levels in culture media did not differ between DMD and control (Fig. 4B)

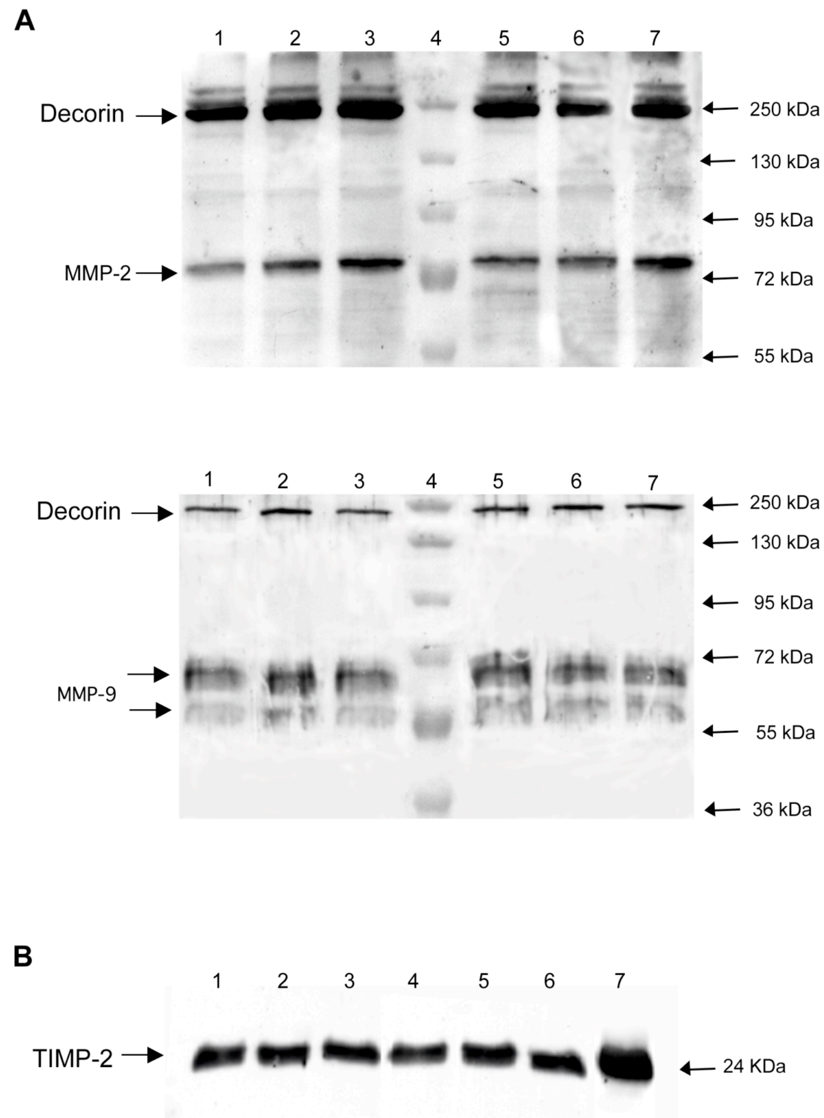


Figure 4. (A) Representative Western blots of decorin, MMP-2 and MMP-9 from DMD and control cell extracts after 21 days of differentiation. Lanes 1-3 control cell extracts; lane 4 molecular weight markers; lanes 5-7 DMD cell extracts. (B) Representative Western blots of TIMP-2 from culture media of DMD and control cultures after 21 days of differentiation. Lanes 1-3 control culture medium; lanes 4-6 DMD culture medium, lane 7 TIMP-2 standard.

Immunocytochemistry

Immunolocalization after 21 days of differentiation did not differ between enriched DMD and control myotube cultures. Decorin and TGF-β1 were expressed in the cytoplasm of myotubes, but not in myoblasts (Fig. 5). Myostatin was observed as small, intensely-stained spots within myotube and myoblast nuclei (Fig. 5); they were significantly ($P<0.01$) more numerous in DMD (2.23 ± 0.26) than control (1.87 ± 0.16) cultures. MMP-2 positivity was observed in the cytoplasm of myotubes with more intense staining close to the nuclear membrane (Fig. 5), although co-localization with emerin (marker of nuclear membrane) showed that MMP-2 was external to the nucleus (Fig. 6). MMP-2 was also observed in fibroblastoid cells; immunostaining with anti-smooth muscle a-actin antibody indicated these cells were myofibroblasts (Tomasek et al., 2002) as most were positive for both proteins (Fig. 6). When counted in randomly selected fields the number of myofibroblast cells was always low and did not differ significantly between DMD and control cultures (controls: 9.52 ± 5.98; DMD 8.69 ± 3.73; $p=0.768$).

MMP-9 was expressed mainly in myotube nuclei with greater intensity at the nuclear rim (Figs. 5 and 6); there was also some immunostaining in the cytoplasm. MMP-9 was never observed in myoblasts. In both control and DMD cultures, TIMP-2 was expressed in the cytoplasm of myotubes, but not in myoblasts (Fig. 5).

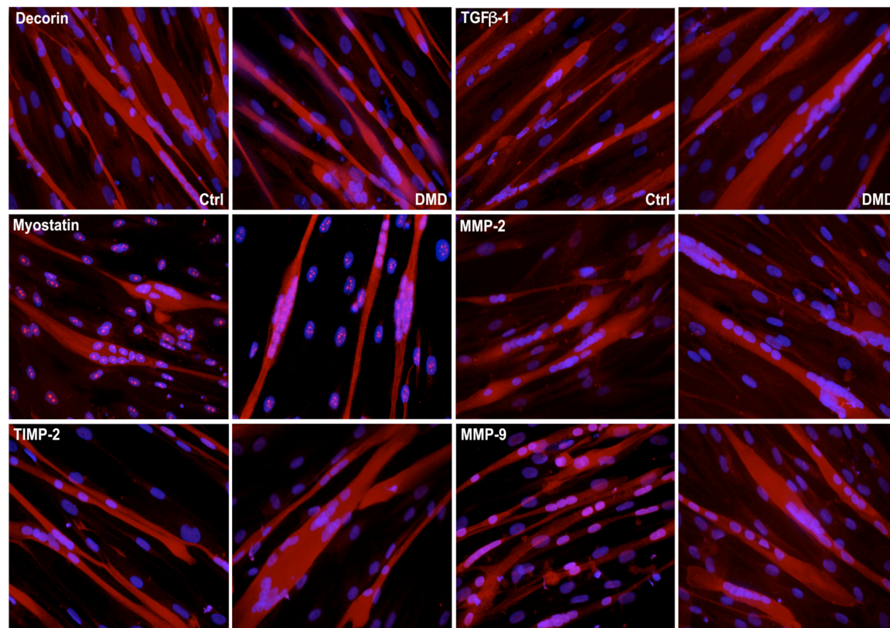


Figure 5. Immunocytochemistry of decorin, TGF-β1, myostatin, MMP-2, TIMP-2 and MMP-9 in control (first and third columns) and DMD (second and fourth columns) myotubes. Scale bar = 25 mm.

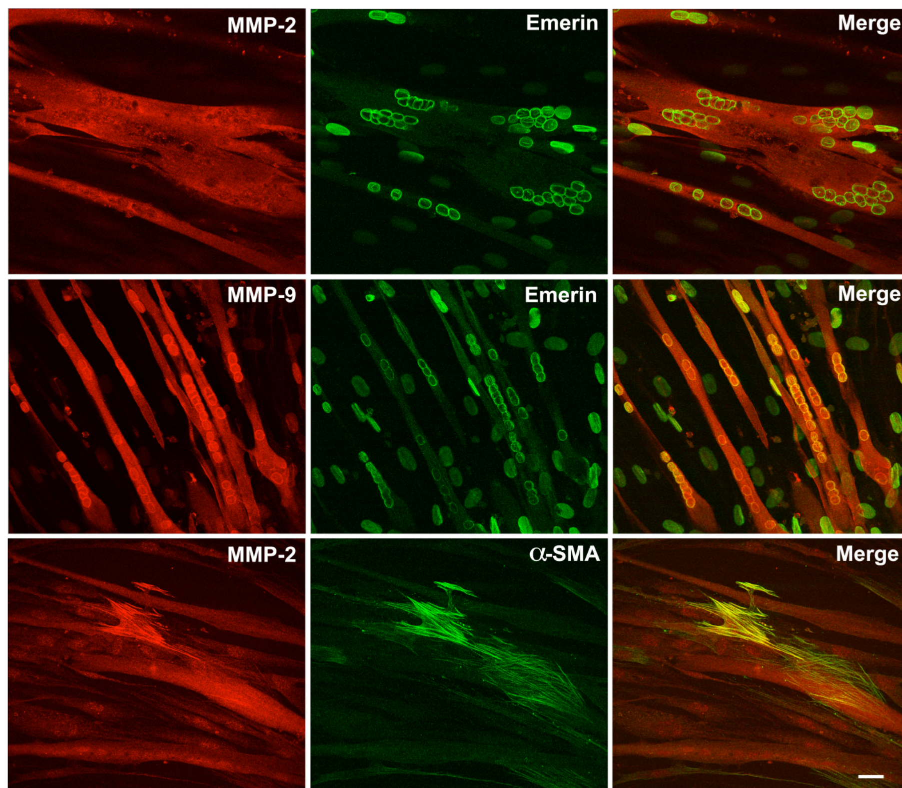


Figure 6. Confocal co-localization of MMP-2 and emerin, MMP-9 and emerin, and MMP-2 and α-SMA in control cultures. Scale bar = 25 mm.

DISCUSSION

We have shown that the expression of decorin transcripts is significantly reduced, and the expression of TGF-β1 transcripts significantly increased, in DMD myotube cultures compared to controls. We have observed similar differences in the expression of these molecules in vivo (Zanotti et al., 2005). However, since the

inflammatory and degenerative processes characterizing DMD muscle in vivo are presumably absent in culture, the changes in transcript expression must arise more directly from the altered DMD muscle cells. It is possible that myofibroblasts also contribute to high TGF-β1 levels, however myofibroblast numbers did not differ between DMD and control cultures.

Transcript expression of the ECM components MMP-2, TIMP-1 and TIMP-2 was also significantly increased in DMD myotube cultures, as was the proteolytic activity of MMP-2. However expression of the proteins decorin, TGF-β1, MMP-2, MMP-9 and TIMP-2 did not differ between DMD and controls. This may be due to differences in turnover time between transcripts and proteins or the fact that many of the proteins are exported to the ECM.

The enhanced activity of MMP-2 in DMD myotube culture medium would probably contribute to deregulated ECM turnover in vivo. Increased MMP-2 transcript levels have also been found in DMD muscle in vivo (unpublished data). We sought to obtain further information on ECM turnover by investigating TIMP activity, but the technique of reverse zymography was insensitive in our hands.

Taken as a whole, our findings suggest that mechanisms regulating ECM homeostasis in muscle tissue are altered in DMD and that the muscle cells contribute fundamentally to this process: By allowing leakage of cellular components to the extracellular space, or abnormal cellular uptake of extracellular growth factors, cytokines, or enzymes, dystrophin absence itself could well exert a direct influence on ECM homeostasis.

Another possible reason for the differences we found between DMD and control cell cultures could be that the expanded cell populations differed. For example the dystrophic cells may have been senescent: they are derived from progenitor satellite cells that undergo many more cell divisions than those in normal muscle in order to provide material for the muscle regeneration cycles that follow repeated muscle degeneration (Webster and Blau, 1990). The significantly greater number of cells expressing the myogenic marker desmin at seeding suggests that DMD myoblasts were more mature than controls. Senescence could contribute in vivo to the pathophysiology of muscle wasting and to the development of fibrosis. It should be noted, however, that myogenicity and fusion indices were similar in DMD and control cultures indicating under differentiating conditions the myoblasts in both groups in were similar.

It is also possible that the progenitor satellite cells could have been differently 'conditioned' in vivo in DMD and control muscle by cytokines or growth factors, although this seems unlikely to have contributed to the differences we found, since myoblasts (derived from satellite cells) are reprogrammed to proliferate and differentiate like embryonic myoblasts (Bischoff, 1986).

Another important finding of our study was that both transcript and protein levels of myostatin were significantly higher in DMD cultures than controls. Myostatin is a potent inhibitor of satellite cell activation and cell renewal (McCroskery et al., 2003). Spontaneous inactivating mutations in the myostatin gene (*MSTN*) in mice (Varga et al., 1997), cattle (McPherron and Lee, 1997) and humans (Schuelke et al., 2004) result in significantly greater muscle mass due to muscle hypertrophy

and hyperplasia. If *MSTN* expression is increased in DMD cells in vivo it may make a major contribution to the defective growth and repair of muscles in DMD patients. Similarly, inhibition of *MSTN* expression appears as a potential treatment for muscular dystrophies and other muscle wasting diseases. Myostatin also binds to decorin (Miura et al., 2006) a substance known to have antifibrotic effects (Border et al., 1992; Giri et al., 1997; Kolb et al., 2001; Logan et al., 1999; Fukushima et al., 2001).

The recent gene expression profiling study of Pescatori et al. (2007) showed that a dystrophic molecular signature is present in the muscle of DMD patients at less than two years of age, despite very limited muscle dysfunction at this time. This signature, which is similar to that observed in older patients with more severe clinical features, includes altered expression of genes involved in the inflammatory response, ECM remodeling, muscle regeneration and energy metabolism. Our in vitro findings in DMD myotubes appear to be an early expression of this early DMD signature: like Pescatori et al. (2007) we found increases in TGF-β1, MMP-2, TIMP-1 and TIMP-2; however, while we found decreased expression of the decorin gene, consistent with our previous results in DMD muscle tissue, while Pescatori et al. (2007) reported increased decorin expression. To complicate matters, the online data of another study on gene expression profiling suggest that decorin expression is unchanged in DMD (Haslett et al., 2002). We suspect that decorin may be crucially involved in fibrotic proliferation in DMD, however more studies are required to resolve conflicting findings reported so far.

To conclude, our data show that the expression of several ECM components is altered in DMD muscle cells in vitro indicating that these cells contribute fundamentally to ECM dysregulation. Our findings that myostatin – a potent inhibitor of satellite cell activation and cell renewal – is increased, and that decorin – a potent binder and downregulator of TGF-β1 and myostatin – is decreased, may have implications for DMD therapy that aims to reduce muscle fibrosis. Studies on the fibroblasts and myofibroblasts present in muscle cultures and which contribute to ECM formation in vivo, will help to further characterize the fibrotic process in DMD.

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Chapter 4

Altered production of extracellular matrix components by muscle-derived Duchenne muscular dystrophy fibroblasts before and after TGF-β1 treatment.

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ABSTRACT

To probe pro-fibrotic mechanisms in dystrophic muscle, we isolated primary fibroblasts from DMD and control muscle biopsies and induced transdifferentiation to myofibroblasts by TGF-β1 treatment. We compared proliferating activity and soluble collagen production, as well as transcript and protein levels of decorin, myostatin, TGF-β1, MMP-1 (interstitial collagenase), MMP-2 (gelatinase), MMP-3 (stromelysin), MMP-7 (matrilysin), and the MMP inhibitors TIMPs 1 to 4, in fibroblasts and myofibroblasts. Principal differences included: significantly greater proliferation rate and soluble collagen production; significant upregulation of decorin, myostatin and MMP-7 transcripts and proteins; and significant downregulation of MMP-1 and TIMP-3 transcripts (with MMP-1 protein reduced by ELISA and TIMP-3 protein apparently reduced on Western blot), in untreated DMD fibroblasts compared to controls. TGF-β1 transdifferentiation significantly lowered decorin and myostatin, and significantly increased TGF-β1 transcript and protein; significantly increased MMP-1 and TIMP-3, and significantly lowered MMP-7 transcript and protein in DMD cells compared to pretreatment. The several differences between DMD and control fibroblasts show that DMD fibroblasts have a profibrotic phenotype, accentuated by TGF-β1 treatment. Dystrophin absence itself could exert a direct influence on ECM homeostasis by allowing leakage of cellular components to the extracellular space, or abnormal cellular uptake of extracellular growth factors, cytokines, or enzymes to influence muscle fibroblasts either directly by altering adhesion properties or indirectly by

interactions with molecules released into the ECM by muscle or inflammatory cells. The transdifferentiation of muscle fibroblasts may serve as a simplified model of fibrosis to further elucidate mechanisms of muscle fibrosis and test possible anti-fibrotic agents.

INTRODUCTION

Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene on the X chromosome, is one of the most common inherited neuromuscular diseases, affecting 1 in 3500 male births. Dystrophin, the dystrophin-associated glycoprotein complex, and laminin a-2 form a link between the extracellular matrix (ECM) and the intracellular cytoskeleton that is crucial for maintaining the structural integrity of muscle fibers (Matsumura and Campbell 1994; Blake et al. 2002). The main consequences of dystrophin absence in skeletal muscle are sarcolemmal instability and increased fiber vulnerability to mechanical stress, resulting in fiber degeneration, followed to some extent by regeneration. However, complete regeneration is prevented by proliferation of connective tissue (fibrosis) that progressively replaces muscle tissue.

Fibrosis is a complex incompletely understood process characterized

by excessive accumulation of collagens and other ECM components. It occurs in conditions affecting skeletal muscle, liver (Kossakowska et al. 1998), kidney (Schnaper et al. 1998), myocardium (Li et al. 2000; Herpel et al. 2006), lung (Selman et al. 2001) and biliary tract (Milani et al. 1990).

The extensive structural disorganization and remodeling that characterize the fibrotic process involve imbalance of matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs), as well as release of fibrogenic cytokines including transforming growth factor-β1 (TGF-β1) (Mauviel 2005), and alteration of proteoglycans such as decorin and biglycan (Westergren-Thorsson et al. 1993). Numerous functions of decorin have been documented, including protein-protein interactions, cell adhesion, signal transduction and DNA repair (Vogel et al. 1984; De Luca et al. 1996; Iozzo 1999). Functions of likely importance in muscle diseases include binding to collagens, TGF-β1, and myostatin (Yamaguchi et al. 1990; Hildebrand et al. 1994; Miura et al. 2006). Myostatin regulates the growth of myocytes (McPherron et al. 1997). Recent work has shown that myostatin also directly stimulates the proliferation of muscle fibroblasts and the production of ECM proteins in vitro and in vivo and that muscle fibroblasts express myostatin and its putative receptor (Li et al. 2008).

TGF-β1 intervenes in the regulation of ECM composition through stimulation of protein production, inhibition of matrix degradation, and control of adhesion proteins required for cell-matrix interactions (Montesano and Orci 1988, Kissin et al. 2002). Its aberrant expression has been implicated in fibrotic and inflammatory conditions in kidney,

liver, and lung (Border and Ruoslahti 1992; Sime et al. 1997; Iredale 2007). In muscle tissue from dystrophin- and *LAMA2*-mutated muscular dystrophy patients, TGF-β1 transcript levels have been shown to be greatly increased (Bernasconi et al. 1995; Zanotti et al. 2005). However, the molecular mechanisms involved in the profibrotic role of TGF-β1 are not fully understood. The cytokine is a direct inducer of the myofibroblast phenotype (Desmoulière et al. 1993; Ronnov-Jessen and Petersen, 1993). Myofibroblasts occur in wound healing and fibrosis (Sappino et al. 1990; Gabbiani 2003) and play a major role in these processes because of their ability to synthesize various ECM components such as fibronectin and collagen (Ignotz and Massagué 1986), growth factors, cytokines (Finlay et al. 2000), growth factor receptors (Thannickal 1995) and integrins (Heino 1989) involved in the repair and remodelling of connective tissue. Normally, when tissue repair is completed, myofibroblasts disappear, probably as a result of apoptosis, and normal tissue function is restored (Desmoulière 1995). However, if the tissue repair program is not terminated appropriately, myofibroblasts persist in the lesion and may give rise to scarring and chronic fibrotic conditions (Lorena 2002; Desmoulière 2005).

In order to increase understanding of pro-fibrotic mechanisms in dystrophic muscle, we isolated primary human fibroblasts from DMD and control muscle biopsies, and induced them to differentiate into myofibroblasts with TGF-β1. We investigated, in both fibroblasts and myofibroblasts, proliferating activity and soluble collagen production, as well as transcript and protein levels of decorin, myostatin, TGF-β1, MMP-1 (interstitial collagenase), MMP-2 (gelatinase), MMP-3

(stromelysin), MMP-7 (matrilysin), and the MMP inhibitors TIMP-1, TIMP-2, TIMP-3 and TIMP-4.

MATERIALS AND METHODS

Cell cultures

Quadriceps muscle biopsies were obtained after informed parental consent from 4 DMD patients (aged 1-7 years) and 4 controls (aged 1-10 years) suspected of neuromuscular disease, but who had normal muscle on biopsy. Investigations on human tissue were approved by our institutional review board. DMD was diagnosed by dystrophin testing and gene analysis.

Primary fibroblasts were derived from muscle biopsies by immunoselection from the myogenic lineage (Zanotti et al. 2007). After culturing in Dulbecco's modified Eagle's medium (DMEM, Cambrex Corporation, East Rutherford, NJ) containing 20% heat-inactivated fetal bovine serum (FBS) (Cambrex), 1% penicillin-streptomycin (Cambrex), 2 mM L-glutamine (Cambrex), 10 mg/ml insulin (Sigma Aldrich, St. Louis, MO), 2.5 ng/ml basic fibroblast growth factor (bFGF) (Gibco Life Technologies, Carlsbad, CA), and 10 ng/ml epidermal growth factor (EGF) (Gibco) (M growth medium), myoblasts and fibroblasts were separated in a midiMACS cell separator (Milteny Biotec, Bergisch Gladbach, Germany), using immunomagnetic beads to which the anti-human CD56 surface marker (Dickson et al. 1987) was attached. After allowing CD56-negative cells (fibroblasts) to pass through the column, CD56-positive cells (myoblasts) were flushed out using the plunger. CD56-positive and negative cell fractions were subsequently seeded onto collagen-

coated Petri dishes: fibroblasts were grown in DMEM containing 10% FBS, 1% penicillin-streptomycin and L-glutamine (F growth medium), while myoblasts were grown in M growth medium and frozen for future analysis.

Myofibroblasts were obtained by treatment with TGF-β1 as follows: Fibroblast growth was first arrested by serum withdrawal, rinsing with PBS and replacement with medium without serum. Forty-eight hrs later the cells were stimulated by addition of 10 ng/ml h-recombinant TGF-β1 (Peprotech EC, London) for 48 hrs. The cellular response to TGF-β1 was evaluated by quantitating α-smooth muscle actin (α-SMA) transcripts by real time PCR, and α-SMA protein by Western blot and immunocytochemistry using a commercial monoclonal antibody (Sigma Aldrich).

Immunocytochemistry

Fibroblasts and myofibroblasts were fixed in 4% paraformaldehyde, permeabilized in PBS 0.1% Triton-X 100 for 30 min, and incubated overnight with one of the following mouse monoclonals: anti-prolyl-4-hydroxylase (anti-fibroblast), anti-desmin (both from Dako, Copenhagen, Denmark; 1:500 and 1:200, respectively), anti-CD44, anti-CD31, anti-α-SMA (all from Sigma; all diluted 1:200), or anti-collagen I (polyclonal from Chemicon, diluted 1:250). The cells were then incubated with goat anti-mouse Alexa 488 (1:2000, Invitrogen) for 2 hrs; followed by DAPI (Sigma) 1:3000. As negative controls primary antibody was omitted.

Phalloidin staining to demonstrate F-actin was performed on paraformaldehyde-fixed cells by incubation in FITC-phalloidin (Sigma) diluted 1:1000 for 40 min, either in combination with α-SMA

or collagen I immunostaining. Cells were examined under a Zeiss Axioplan fluorescence.

Cell proliferation assay

Cells were plated onto 96-well plates (10^4 cells/well) and incubated overnight with 100 µl F growth medium. Growth medium was then replaced with serum-free medium for 24 hrs, to synchronize the cells; cell proliferation was then determined by measuring incorporation of 5-bromo-2'-deoxyuridine (BrdU), after 4 hrs' incubation, with an enzyme immunoassay kit (BrdU cell proliferation assay kit, Roche Diagnostic, Penzberg, Germany), according to the manufacturer's instructions. The enzymatic reaction was stopped with 25 µl of 2N sulfuric acid, and absorbances were read at 450 nm on a Victor Wallac 1420 multi-label reader (Perkin-Elmer, Waltham, MA).

Collagen measurements

Total soluble (non cross-linked) collagen was determined in culture supernatants by a quantitative dye-binding method using the Sircol collagen assay (Biocolor, Belfast, N. Ireland) according to the manufacturer's instructions.

cDNA synthesis

Total RNA was isolated from fibroblasts or myofibroblasts using TRI Reagent (Ambion, Inc. Austin, TX) according to the manufacturer's instructions and checked spectrophotometrically for quantity and purity. One µg aliquots of RNA were reverse transcribed in the presence of 5x first strand buffer (Invitrogen), 1 mM of each deoxynucleoside triphosphate, 8 pM random hexamers, 10 µM dithiothreitol, 1 IU/µl RNAse inhibitor (Roche Molecular Biochemicals, Basel, Switzerland) and 10 IU/µl M-MLV reverse

transcriptase (Invitrogen) with incubation at 37°C for 1 h and at 95°C for 5 min. The reaction product was stored at -20°C pending use. cDNA integrity was assessed by PCR amplification of human GAPDH (GenBank accession no. M33197) using specific primers (forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'). PCR conditions were: 94°C 1 min, 54°C 1 min, 72°C 1 min, for 35 cycles.

Real time PCR

Target gene expression was analyzed by quantitative real time PCR. TaqMan Universal PCR MasterMix and Assays-on-Demand Gene Expression probes (Applied Biosystems, CA) (see Table 1) were used for the PCR step. Reactions were performed in 96-well plates with 25 ml volumes. All samples were analyzed in triplicate. Cycling parameters were: 2 min at 50°C, 95°C for 10 min, and followed by 40 cycles of PCR (15 s at 95°C and 1 min at 60°C). Products were detected with the ABI Prism 7000 sequence detection system (Applied Biosystems). The expression of each target gene in control and DMD fibroblasts was normalized to the expression of β-actin and determined as the ratio of the target gene to β-actin gene calculated by $2^{-\Delta Ct}$, where $\Delta Ct = Ct^{Target} - Ct^{\beta\text{-actin}}$. Differences in gene expression between basal and TGF-β1 treated control and DMD cells were calculated using the mathematical model based on PCR efficiency (E), described by Pfaffl (2001). According to this model, differences in expression were determined by the equation $E^{\Delta CT\text{gene basal} - \Delta CT\text{ gene TGF-}\beta 1\text{ stimulated}}$.

Target mRNA	GeneBank accession	Assay-on-Demand kit	Amplicon Lenght (bp)
Decorin	**NM_001920**	Hs00266491_m1	59
Myostatin	**NM_005259**	Hs00193363_m1	118
TGF-β1	**NM_000660**	Hs00171257_m1	63
MMP-1	**NM_002421**	Hs00233958_m1	133
MMP-2	**NM_004530**	Hs00234422_m1	83
MMP-3	**NM_002422**	Hs00968305_m1	126
MMP-7	**NM_002423**	Hs00159163_m1	101
TIMP-1	**NM_003254**	Hs00355335_g1	61
TIMP-2	**NM_003255**	Hs00234278_m1	73
TIMP-3	**NM_000362**	Hs00165951_g1	76
TIMP-4	**NM_003256**	Hs00162784_m1	72

Table 1. Assays-on-Demand Gene Expression probes

Protein extraction

Cells were washed with PBS and lysed in extraction buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet-P, 1% Triton-X 100) containing a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA) for 30 min on ice. The lysate was centrifuged at 18,000 g for 15 min at 4°C. The supernatant was collected and protein concentration determined using the DC Protein Assay Reagent (Bio-Rad).

Zymography

MMP-1 and MMP-2 activities were detected in the medium after 5x concentration using an Ultrafree-4 centrifugal filter unit (Millipore Corporation, Billerica, MA, USA) and protein concentration was determined using the DC Protein Assay Reagent.

Ten μg of 5x concentrated samples were then diluted 1:1 in non-reducing buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 0.1% bromophenol blue) and electrophoresed on 7.5% SDS-polyacrylamide gels containing 1 mg/ml either b-casein (for detection of MMP-1 activity) or gelatin type A from pig skin (for detection of MMP-2 activity) (both from Sigma). The gels were washed twice for 15 min in 2.5% (v/v) Triton X-100, rinsed with distilled H_2O at room temperature, and incubated overnight in developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 200 mM NaCl) at 37°C. The gels were then stained with 0.5% Coomassie brilliant blue R-250 (in 30% methanol, 10% acetic acid) for 3 hrs and de-stained with aqueous methanol (30%) and acetic acid (10%). Areas where the gelatin had been degraded by proteolytic activity were seen as absence of staining.

Western blot

For detection of α-SMA, 10 μg of cell protein extracts, were solubilized in 2x Laemmli buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 1% bromophenol blue), boiled, separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell Inc., Keene NH, USA). Membranes were probed with anti-α-SMA, diluted 1:250, and anti-β-tubulin (internal standard), diluted 1:2500 (both monoclonal

antibodies from Sigma). Biotin-conjugated secondary antibody was then applied (1:2500; Jackson ImmunoResearch, Westgrove PA, USA), followed by peroxidase-conjugated streptavidin (1:3000; Jackson ImmunoResearch), and by detection with the ECL chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

MMP-2, MMP-3, TIMP-1, TIMP-2, and TIMP-3, all secreted extracellularly, were detected in supernatants prepared and concentrated as described above. Ten μg of supernatant proteins, were solubilized in 2x Laemmli buffer, boiled, separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with one of the following rabbit polyclonal antibodies: anti-MMP-2, anti-TIMP-2 (both from Biomol), anti-MMP-3, anti-TIMP-1, anti-TIMP-3 (Immunological Sciences, Rome, Italy), all diluted 1:250, and detected as described above.

For detection of decorin, 20 μg of cell protein extract or cell medium were digested with 25 mU chondroitinase ABC (Sigma) for 6 hrs at 37 °C. Samples were then solubilized in 2x Laemmli buffer, boiled, electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membranes. These were probed with goat polyclonal anti-decorin (Calbiochem, Merck Chemicals, Nottingham, UK; 1:100) and revealed as described above.

ELISA

MMP-1, MMP-7, TGF-β1, and myostatin in the extracellular medium were not detected by Western blot and were therefore evaluated by ELISA. MMP-1 was detected by the Calbiochem sandwich ELISA assay according to the manufacturer's instructions, while MMP-7,

TGF-β1 and myostatin were detected with direct ELISA assays. Briefly, plates were coated with 100 ml of medium or protein standards (for calibration curve) and incubated at 4°C overnight. Unbound antigen was removed from the plates by tapping off the liquid, and several PBS 0.1% Triton-X 100 washings. To block non-specific binding, 200 μl of 1% BSA/PBS were added for 2 hrs at room temperature, followed by several washings as above. One hundred μl of polyclonal anti-MMP-7 (Biomol), or monoclonal anti-TGF-β1 (BioSource International, Inc. Camarillo, CA), or polyclonal anti-myostatin (Chemicon), all diluted 1:100, was then added and incubated overnight at 4 °C. After several washings, the wells were incubated with the second step alkaline phosphatase-conjugated antibody for 2 hrs, followed by repeated washings, incubation with 100 μl of substrate solution for 1 hr and, finally, addition of stopping solution. The wells were then read on an ELISA Victor Wallac 1420 multi-label plate reader.

Statistical analysis

All experiments, except Western blots, represent data from at least 3 patients and 3 controls, always performed at least 9 times. For the Western blots, concentrated supernatants from 3 or 4 patients were pooled, as were those from at least 3 controls.

For RT-PCR, because of inherent variability in data from different cell isolates, all quantitative data in basal conditions were expressed as ‘fold’ changes relative to control levels expressed as 1. Quantitative RT-PCR data after TGF-β1 treatment were expressed as fold changes relative to basal values.

The results were expressed as means and standard deviations. Differences between the two groups (DMD and control) were assessed using the Wilcoxon non-parametric test and considered significant for $p \leq 0.05$.

RESULTS

Cell characterization

All CD56-negative cells from both DMD and controls immunostained with anti-prolyl 4-hydroxylase (Figs. 1a and 1b) and were therefore fibroblasts. Furthermore, none of the cells immunostained with anti-CD44 (marker of interstitial cells) or anti-CD31 (marker of endothelial cells); while less than 1% of the cells were immunopositive for desmin (marker of myogenic cells) both in DMD and control populations (data not shown). We therefore conclude that our fibroblast populations were homogeneous and comparable.

After treatment with TGF-β1, FITC-phalloidin staining showed marked modification of the cytoskeletal protein F-actin both in DMD and control fibroblasts, evident as formation of new stress fibers (Fig. 2); α-SMA immunostaining showed considerably increased expression of α-SMA in the stress fibers, more intense in DMD than in control cells (Fig.1c). Collagen I immunostaining showed greater positivity in DMD fibroblasts than control fibroblasts, after TGF-β1 treatment (Fig. 2).

α-SMA transcript levels in DMD fibroblasts did not differ significantly [0.51 ± 0.25 ($p = 0.10$)] from those in control fibroblasts (considered as 1). After TGF-β1 treatment, both DMD [7.78 ±1.96 ($p = 0.02$)] and control [5.38 ± 1.80 ($p = 0.02$)] myofibroblasts expressed

significantly increased α-SMA transcript levels compared to basal levels (Fig. 1d). The difference between DMD and control myofibroblasts was also significant ($p = 0.03$).
By western blot, the band corresponding to α-SMA increased in intensity after TGF-β1 treatment both in DMD and control cells (Fig. 1d).
From these data we conclude that after TGF-β1 treatment our cell populations show features characteristic of myofibroblasts. From now on we refer to cells before TGF-β1 treatment as fibroblasts (although few myofibroblasts were present in both cell populations) and to those after TGF-β1 treatment as myofibroblasts.
The cell proliferation assay showed that DMD fibroblasts and myofibroblasts incorporated significantly more BrdU than control cells. Absorbances were: 0.27 ± 0.04 vs. 0.24 ± 0.06 ($p = 0.001$) in fibroblasts and 0.34 ± 0.06 vs. 0.29 ± 0.04 ($p = 0.001$) in myofibroblasts; TGF-β1 treatment caused a further significant increase in BrdU incorporation compared to basal values both in DMD and control cells ($p = 0.001$) (Fig. 1e) (values were means of 4 independent experiments each including 3 cell lines seeded in 4-6 wells for a total of 16-24 determinations per condition).
Soluble collagen production in the culture medium was significantly greater in DMD fibroblasts than normal fibroblasts: 124.28 μg/ml ± 56.16 vs. 28.87 μg/ml ± 13.45, ($p = 0.001$) (Figs.1d and 6). TGF-β1 treatment caused a further significant increase in total soluble collagen production in both cell populations: 268.05 μg/ml ± 63.96 in DMD vs. 175.47 μg/ml ± 44.70 in control myofibroblasts. Values differed significantly both between groups and before and after

transdifferentiation to myofibroblasts (p = 0.01) (Fig. 1f).

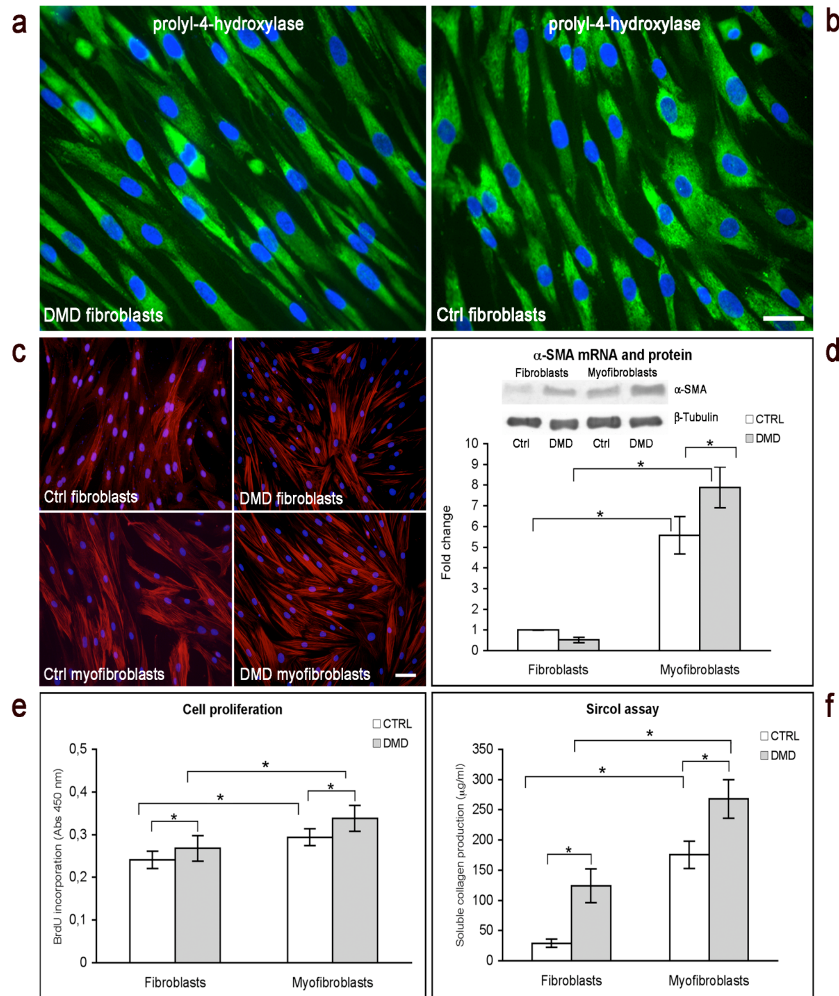

Fig. 1 (a, b) Immunostaining with anti-prolyl-4-hydroxylase and DAPI shows that DMD and control (Ctrl) fibroblast cultures contain fibroblasts exclusively; (c) anti-α-SMA immunostaining shows

considerably increased expression of α-SMA in the stress fibers of myofibroblasts both in control and DMD cells. Bar: 20 mm. (d) Real time PCR and Western blot show that TGF-β1-treated cells produce abundant α-SMA which is present in low levels in untreated cells. (e) Cell proliferation assay as determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation shows significantly greater proliferation rate in DMD than control fibroblasts and myofibroblasts and significantly greater proliferation rate in TGF-β1 treated cells compared to untreated cells. (f) Total soluble collagen as measured by the colorimetric Sircol assay shows significantly greater collagen concentration in media of DMD fibroblast and myofibroblast cultures than in media of respective normal cultures.

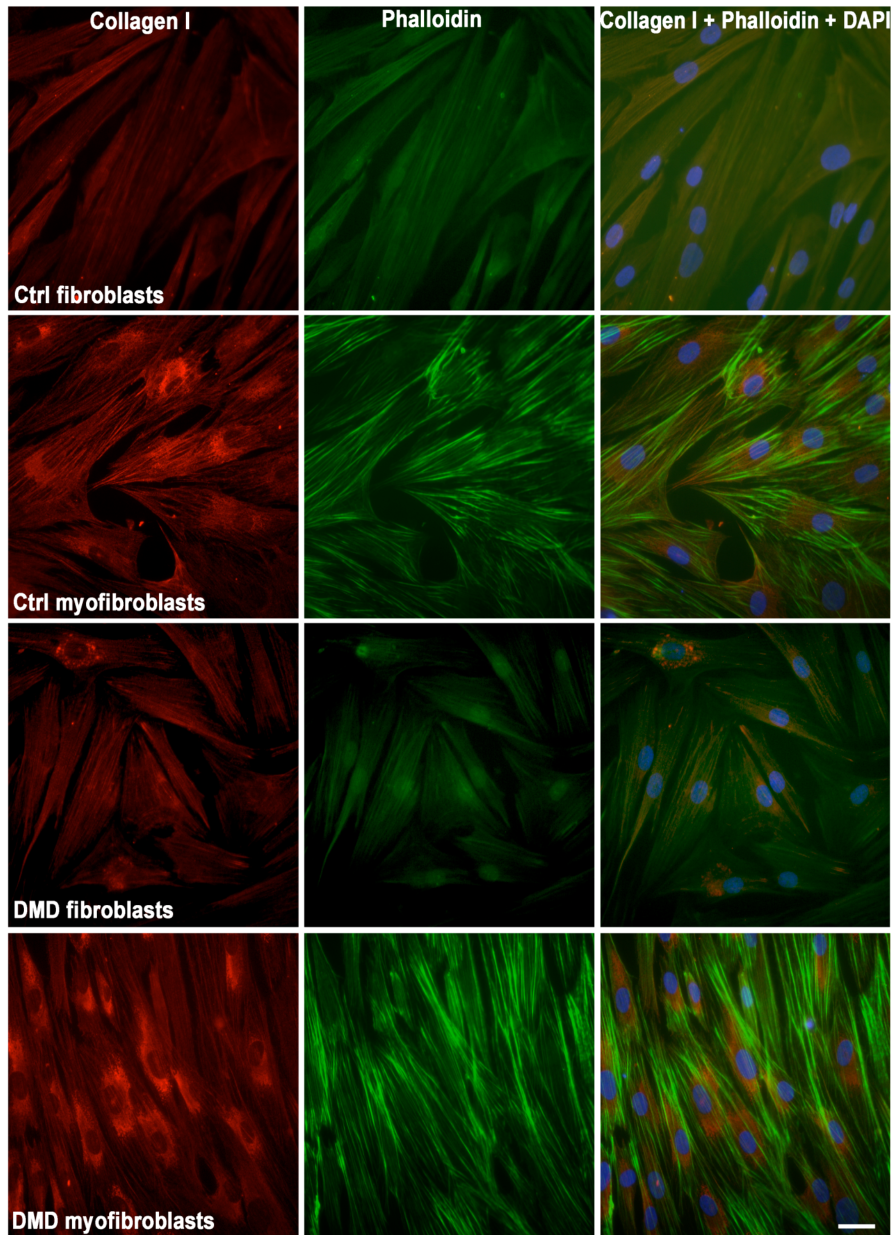

Fig. 2 Colocalization of collagen I (by immunostaining) and F actin (by phalloidin staining) showing greater positivity for collagen I and more stress fibers in DMD myofibroblasts than controls. Scale bar: 20 μm.

Real time PCR

DMD fibroblasts expressed significantly more myostatin [4.86 ± 1.73 (p = 0.02)], and decorin [2.35 ± 0.54 (p = 0. 010)] mRNA than controls, while TGF-β1 [0.79 ± 0.25 (p = 0.06)] did not differ significantly (Fig. 3a).

In DMD myofibroblasts, TGF-β1 treatment significantly lowered transcript levels (compared to basal) of decorin [0.14 ± 0.03 (p = 0.02)] and myostatin [0.14 ± 0.06 (p = 0.02)], and increased TGF-β1 [1.97 ± 0.55 (p = 0.02)]. In controls, differences were not significant [(decorin: 0.11 ± 0.08 (p = 0.07); myostatin: 0.42 ± 0.32 (p = 0.07); TGF-β1: 1.92 ± 0.69 (p = 0.07)]. (Fig.3b).

DMD fibroblasts expressed significantly less MMP-1 [0.18 ± 0.10 (p = 0.05)] (Fig. 4a) and significantly more MMP-7 [7.75 ± 1.83 (p = 0.05)] (Fig. 4b) transcripts than controls. After TGF-β1 treatment MMP-1 levels increased significantly [5.23 ± 2.23 (p = 0.02)] (Fig. 3a) and MMP-7 levels decreased significantly in DMD myofibroblasts [2.92 ± 1.13 (p = 0.02)] (Fig. 4b), compared to basal values, while in controls neither MMP transcripts changed significantly following treatment [MMP-1: 0.50 ± 0.36 (p = 0.19); MMP-7: 1.91 ± 0.46 (p = 0.07)].

TIMP-3 transcript levels [0.55 ± 0.11 (p = 0.02)] were significantly lower in DMD fibroblasts than controls (Fig. 5a) and increased significantly after TGF-β1 treatment, both in DMD [3.85 ± 1.13 (p = 0.01)] and controls [6.81 ± 2.16 (p = 0.02) (Fig. 5b).

MMP-2, MMP-3, TIMP-1, TIMP-2 and TIMP-4 transcript levels did not differ significantly between DMD and control cells before TGF-

β1 treatment, and did not change significantly after TGF-β1 (data not shown).

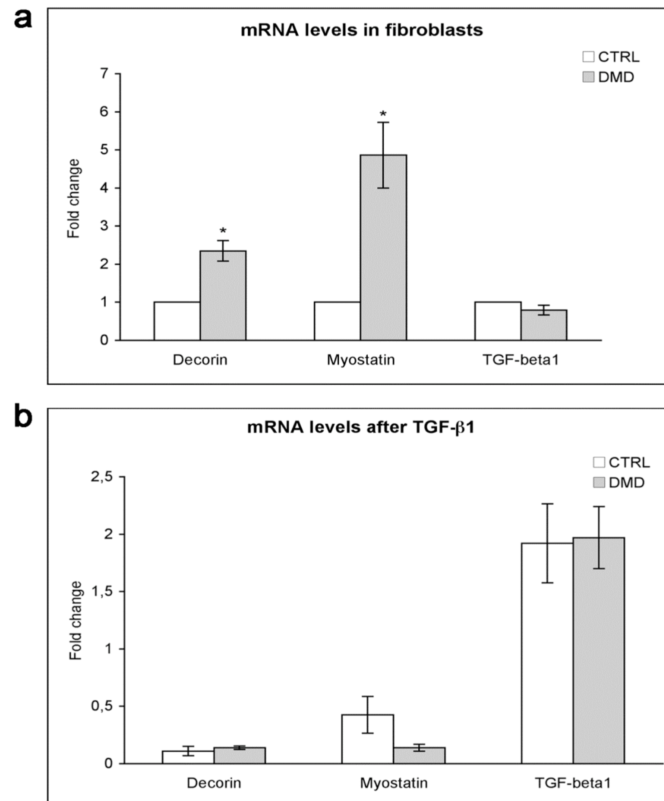

Fig. 3 (a) Real time PCR of decorin, myostatin and TGF-β1 shows significantly higher levels of decorin and myostatin transcripts, but not significantly different TGF-β1 transcript levels in DMD than control fibroblasts. (b) TGF-β1 treatment significantly lowered decorin and myostatin and significantly increased TGF-β1 transcript levels in DMD compared to pretreatment; changes in controls were not significant.

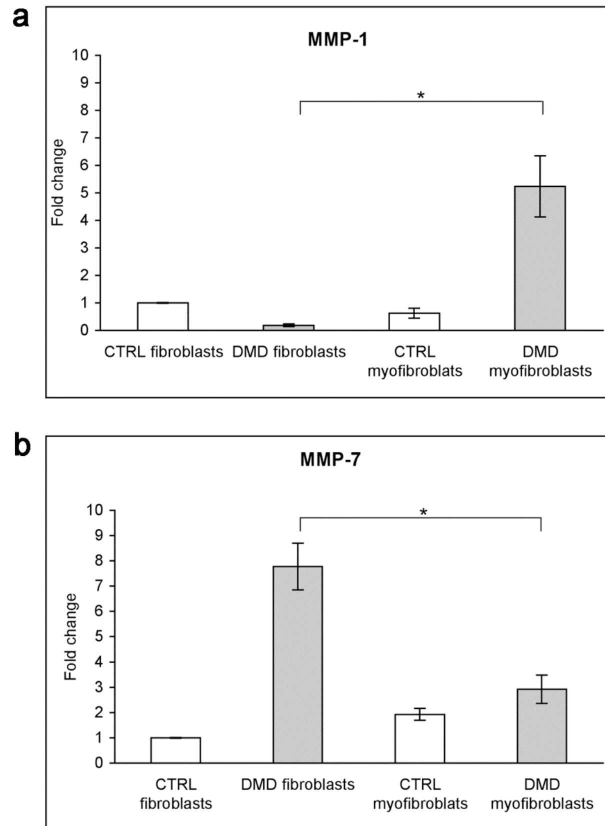

Fig. 4 Real time PCR of MMP-1 (a) and MMP-7 (b) shows that DMD fibroblasts had significantly lower transcript levels of MMP-1, and significantly higher MMP-7 levels than control fibroblasts. TGF-β1 treatment significantly increased MMP-1 (b) transcripts and significantly lowered MMP-7 (b) transcripts in DMD compared to pretreatment; changes in controls were not significant

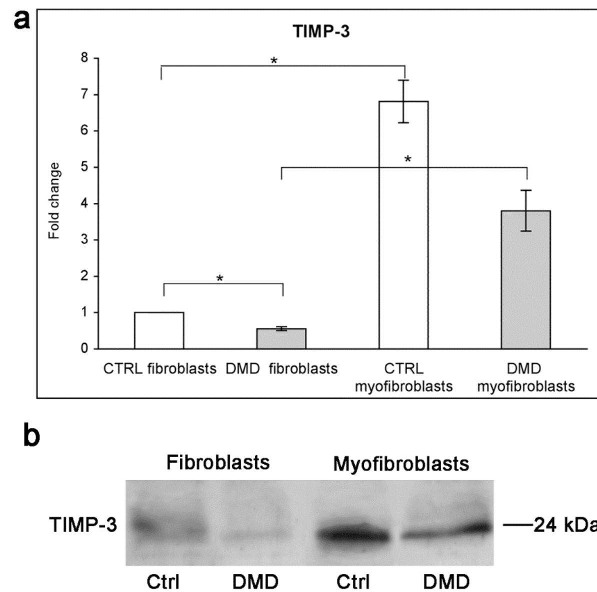

Fig. 5 Real time PCR (a) and Western blot (b) of TIMP-3. (a) TIMP-3 transcript levels were significantly lower in DMD fibroblast cultures and that TGF-β1 treatment significantly increased transcript levels in both DMD and controls. (b) The TIMP-3 protein band was more prominent in treated DMD and control cells compared to untreated cells.

Zymography

For MMP-1, only in the medium of DMD fibroblasts after TGF-β1 treatment showed positivity: presence of a single band at 48 kDa corresponding to the active form of MMP-1 (Fig. 6).

MMP-2 zymography showed the presence of a 66 kDa gelatinolytic band (inactive form of MMP-2) in medium from fibroblasts, that was more intense in DMD than controls. The 62 kDa band (active form) was absent or barely discernible in DMD and control culture media.

TGF-β1 treatment reduced the intensity of the 66 kDa band, while the 62 kDa band was no longer discernible (Fig. 6).

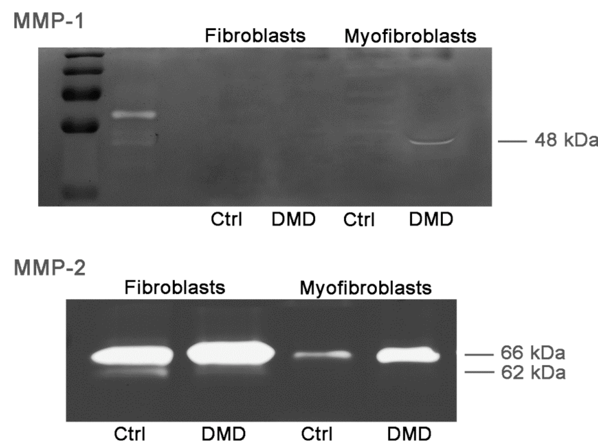

Fig. 6 MMP-1 zymography shows a single band at 48 kDa corresponding to the active form of the MMP-1 in DMD myofibroblasts but not in fibroblasts. MMP-1 is absent from control cells. First 2 lanes on the left: protein standard and positive control for MMP-1. MMP-2 and MMP-3, respectively.
MMP-2 zymography shows a 66 kDa gelatinolytic band corresponding to the inactive form of MMP-2, more intense in DMD fibroblasts than in controls, while the 62 kDa band corresponding to the active form is absent or barely discernible both in DMD or control cultures. TGF-β1 treatment reduced the intensity of the 66 kDa band while the 62 kDa band was no longer visible.

Western blot

Visual inspection of Western blots from cell media (control and DMD samples loaded with equal quantities of total protein) suggested that the intensities of the MMP-2, MMP-3, TIMP-1, and TIMP-2 bands were similar in controls and DMD, both before after TGF-β1 treatment. The TIMP-3 band was more intense in controls than DMD both before and after TGF-β1 treatment (Fig. 5b).

The intensity of the decorin core protein band, evaluated in cell protein extracts, was greater in DMD than control fibroblasts. After TGF-β1 treatment, band intensity reduced markedly in DMD myofibroblasts, and was no longer discernible in control myofibroblasts (Fig. 7a).

In undigested cell media, decorin appeared as a smear at 95-130 kDa; intensity was greater in DMD than controls before TGF-β1 treatment, and similar in both after TGF-β1 treatment. After chondroitinase treatment (leaves the decorin core protein) of fibroblast media, the decorin band at approximately 45 kDa was more intense in media from DMD fibroblasts than controls; the core protein bands were of similar intensity after TGF-β1 treatment (Fig. 7b).

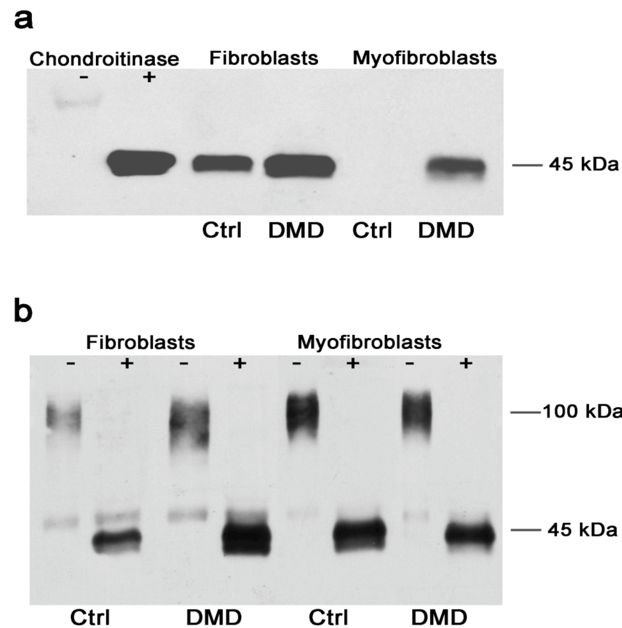

Fig. 7 (a) Western blot of decorin from DMD and control fibroblast and myofibroblast cell extracts showing that TGF-β1 treatment reduced the intensity of decorin core protein band in DMD, while it was no longer visible in control. Lane 1: undigested bovine decorin standard; lane 2: digested bovine decorin standard; lanes 3-6: fibroblast and myofibroblast cell extracts. (b) Western blot of decorin from DMD and control fibroblast and myofibroblast cell media before and after chondroitinase ABC digestion, showing bands of greater intensity in DMD than controls before TGF-β1 treatment, and of similar intensity in both after TGF-β1 treatment.

ELISA

MMP-1 protein levels were significantly lower [0.037 ± 0.004 ng/ml

vs. 0.096 ± 0.018 ng/ml ($p = 0.01$)] (Fig. 8a) and MMP-7 protein levels were significant higher [2.040 ± 0.439 ng/ml vs. 0.993 ± 0.135 ng/ml ($p = 0.001$)](Fig. 8b) in DMD fibroblast medium than control fibroblast medium. TGF-β1 protein levels [0.374 ± 0.144 ng/ml vs. 0.059 ± 0.019 ng/ml ($p = 0.001$)] (Fig. 8c) and myostatin protein levels [2.368 ± 0.349 ng/ml vs. 1.818 ± 0.20 ng/ml ($p = 0.03$)] were also significantly higher in DMD fibroblast medium than control medium (Fig. 8d).

After TGF-β1 treatment, there was a significant increase, compared to basal, in MMP-1 secretion both in DMD [0.60 ± 0.07 ng/ml ($p=0.0003$)] and control media [0.20 ± 0.01 ng/ml ($p=0.019$)] (Fig. 8a). TGF-β1 treatment also caused: a significant decrease in MMP-7 protein levels in DMD cells [1.20 ± 0.30 ng/ml ($p = 0.007$)], but not in controls [1.19 ± 0.25 ng/ml ($p = 0.13$)] (Fig. 8b); a significant decrease in TGF-β1 protein levels in DMD [0.09 ± 0.01 ng/ml ($p = 0.001$)] and significant increase in controls [0.11 ± 0.03 ng/ml ($p = 0.007$) (Fig. 8c); and a significant decrease in myostatin protein levels in both populations [DMD: 0.78 ± 0.14 ng/ml ($p = 0.006$); controls: 1.13 ± 0.20 ng/ml ($p = 0.001$)] (Fig. 8d).

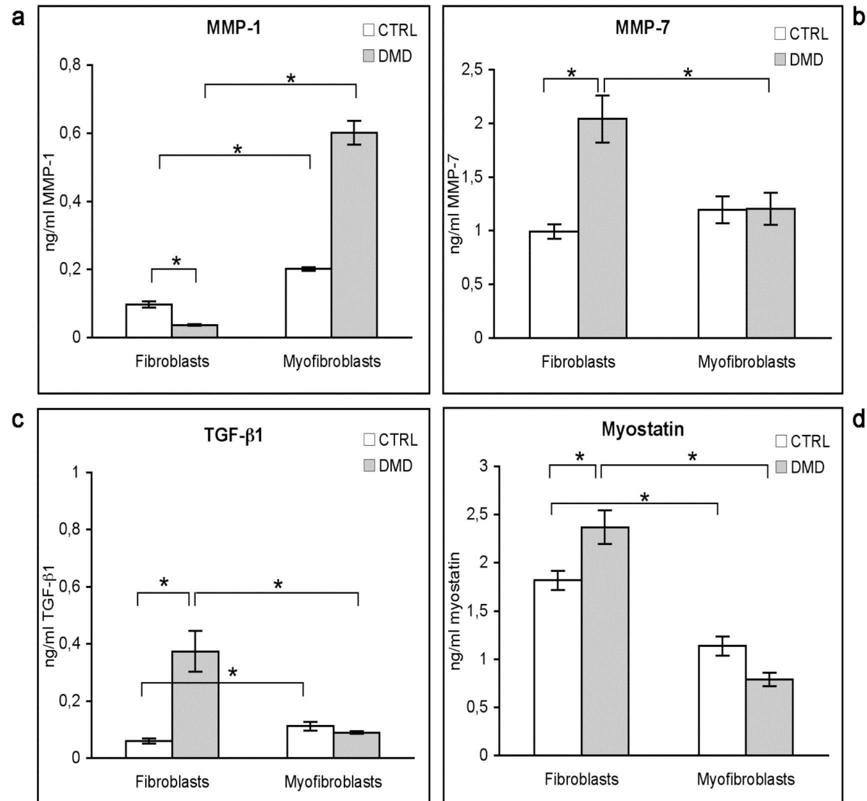

Fig. 8 ELISA of MMP-1 (a), MMP-7 (b), TGF-β1 (c) and myostatin (d), all from media. MMP-1 was significantly lower; MMP-7, TGF-β1 and myostatin were significantly higher in DMD fibroblast media than control media. TGF-β1 treatment increased MMP-1 significantly in DMD, but not controls; and significantly decreased MMP-7, TGF-β1 and myostatin in DMD, but not controls.

DISCUSSION

Fibroblasts play a central role in maintaining the integrity and

composition of the ECM by synthesizing and degrading ECM components. There are several distinct functional subtypes of fibroblasts distinguished by anatomical origin and gene expression profile (retained in culture) (Chang et al. 2002).

Our study shows that primary fibroblasts derived from DMD muscle biopsies differ from control fibroblasts in a number of respects. First, DMD fibroblasts proliferate at a faster rate than control fibroblasts. Since difference increased proliferation rate has been observed in fibroblasts isolated from fibrotic lung compared to control lung (Jordana et al. 1988; Raghu et al. 1988) and in fibroblasts isolated from hypertrophic scar tissue compared to those from normal skin (Zhang et al. 2007).

The second difference between DMD and control fibroblasts is that the production of soluble collagens by DMD fibroblasts was significantly higher than in controls. Similar increases in collagen production have been found in several fibrotic conditions including idiopathic pulmonary fibrosis (Ramos et al. 2001) and cultured fibroblasts from scleroderma (Pannu et al., 2006).

Third, we found a significant increase in decorin transcripts in DMD fibroblasts and also observed an apparent increase in decorin protein both in cell extracts and media. After production within the cell, the decorin core protein is rapidly glycosylated and secreted. We found that both the digested core protein and the glycosylated form were of higher intensity in DMD fibroblast media than control media. Fadic et al. (2006) also reported increased decorin (and also biglycan synthesis) in muscle-derived fibroblasts from a DMD patient compared to controls. Kuroda and Shinkai (1997) found higher levels

of decorin transcripts in systemic sclerosis fibroblasts, and Westergren-Thorsson et al. (2004) reported that levels of decorin secreted by primary fibroblasts derived from a fibrotic lung were increased. In our previous study on the ECM of primary DMD myotube cultures (practically devoid of fibroblasts), we found that decorin transcript levels were lower than in controls (Zanotti et al. 2007), in accord with our findings in DMD patient muscle (Zanotti et al. 2005). This is in marked contrast to our present finding that decorin transcripts are increased in DMD fibroblasts, and could reflect inherent differences in decorin production between the cell types, or differences between fibrotic and non-fibrotic muscle areas; the inflammation present in DMD muscle in vivo may affect the situation. Further investigation, possibly by laser micro-dissection of DMD muscle samples may clarify what is happening.

Fourth, DMD fibroblasts also produced significantly more myostatin transcripts and protein. The protein was not detected by Western blot, probably due to its low concentration relative to total protein, but was detected by ELISA. Myostatin expression in fibroblasts from injured skeletal muscle was first detected by Yamanouchi et al. (2000) indicating that fibroblasts could be a source of myostatin. More recently, Zhu et al. (2007) showed that myostatin stimulates fibroblast proliferation and induces α-SMA expression, as does TGF-β1; while Li et al. (2008) found that biologically active myostatin was expressed and secreted by muscle-derived fibroblasts. We found up-regulated myostatin mRNA and protein in myotube cultures obtained from the same DMD patients evaluated in the present study (Zanotti et al. 2007) but found reduced myostatin mRNA in their muscle biopsies

(manuscript in preparation). These differences could reflect differences between in vitro and in vivo conditions: The in vitro cell population consists mainly of myoblasts and myotubes, while the inflammatory component is present in vivo but not in vitro. Interactions of myostatin with decorin, TGF-β1, or other regulatory molecules might give rise to a persistent positive autocrine feedback loop that results in over-production of matrix proteins and subsequent fibrosis (Schmid et al. 1998).

Although TGF-β1 transcript levels were unchanged, protein levels were significantly increased in DMD fibroblasts by ELISA. TGF-β1 is finely regulated by interactions with various cellular and extracellular players and by positive autocrine feedback (Schmid et al. 1998). Furthermore, increased levels could in part be due to release of the cytokine from decorin, as it is degraded by (increased activity of) MMP-2.

Another finding of the present study is that MMP-7 transcript and protein levels were increased in DMD fibroblasts. The increase in transcript expression was marked, while the increase in protein expression was less striking but still significant. Like several other secreted proteins, MMP-7 was not detected by Western blot. By the more sensitive ELISA method, its expression was significantly greater in DMD than control fibroblasts. Increased levels of MMP-7 have been found in experimental and pathological fibrotic conditions, such as idiopathic pulmonary fibrosis (Zuo et al. 2002), liver fibrosis (Huang et al. 2005) and tubulointerstitial fibrosis (Surendran et al. 2004). Zuo et al, (2002) demonstrated that *MMP-7*$^{-/-}$ knockout mice were protected from bleomycin-induced fibrosis, showing that this

metalloproteinase plays an important role in the development of pulmonary fibrosis. Huang et al. (2005) showed that MMP-7 expression directly correlated with the progression of liver fibrosis. It is likely, therefore, that the MMP-7 produced by DMD fibroblasts may also be involved in skeletal muscle fibrosis, as also suggested by our finding that MMP-7 appears increased, by immunohistochemistry, in DMD muscle biopsies (manuscript in preparation).

We also found that MMP-1 mRNA and protein levels were significantly lower in DMD fibroblasts than controls. MMP-1 plays an important role in limiting fibrosis by degrading type I and III collagen fibrils (Brinckerhoff et al. 1987). In the early stages of fibrosis a slight increase in collagen III has been reported, whereas collagen I is highly increased and remains the major collagen type later on (Eckes et al. 2000). Takeda et al. (1994) showed that MMP-1 is downregulated and its activity reduced in scleroderma. Furthermore, mice expressing an a1 chain of collagen I with a defective MMP-1 cleavage site, develop fibrotic skin alterations similar to those in scleroderma (Liu et al. 1995). Our data, showing excessive soluble collagen production, increased collagen I expression, and concomitant reduction in MMP-1 expression in DMD fibroblasts, suggest a scenario similar to that observed in systemic scleroderma fibroblasts. The recent paper of Kaar et al. (2008) demonstrated that active MMP-1 can effectively reduce muscle scarring due to its ability to digest collagen; MMP-1 therefore emerges as a possible treatment of fibrosis.

The ultimate finding of the present study was that TIMP-3 transcript levels in DMD fibroblasts were significantly reduced. In addition to its role in counteracting MMPs, TIMP-3 is able to induce apoptosis in

various cell types (Ahonen et al. 1998; Baker et al. 1998). This characteristic has been attributed to TIMP-3's tight binding to the ECM (Yu et al. 2000), which confers the unique ability of inhibiting members of the ADAM (a disintegrin and metalloprotease domain) family of enzymes such as tumor necrosis factor-a-converting enzyme (Amour et al. 1998). It may be, therefore, that DMD fibroblasts are more resistant to apoptosis than normal fibroblasts.

The detailed significance of all the differences we have found between normal and DMD fibroblasts is not clear at present, nevertheless it is clear that DMD fibroblasts are characterized by a pro-fibrotic phenotype. This is further confirmed when fibroblasts transdifferentiate into myofibroblasts by TGF-β1 treatment: Both control and DMD transdifferentiated cells expressed significantly more a-SMA and apparently more collagen I, and produced more soluble collagens than fibroblasts, in addition to assuming the myofibroblast morphology. Nevertheless, the proliferation rate and collagen production of DMD myofibroblasts remained significantly higher than control myofibroblasts. These findings are in agreement with observations in fibrotic conditions including lung disease and liver fibrosis (Gharaee-Kermani et al. 2009; Le Bousse-Kerdilès et al. 2008).

It is likely that DMD fibroblasts are predisposed to a profibrotic phenotype as a consequence of their primary genetic defect. In our previous study in DMD myotubes (Zanotti et al. 2007) we showed that mechanisms regulating ECM homeostasis in muscle tissue were altered and suggested that dystrophin absence itself could exert a direct influence on ECM homeostasis by allowing leakage of cellular

components to the extracellular space, or abnormal cellular uptake of extracellular growth factors, cytokines, or enzymes. Based on the findings of the present study we speculate that dystrophin absence influences the muscle fibroblast subtype either directly by altering its adhesion properties or indirectly by interactions with molecules released into the ECM by muscle or inflammatory cells.

Myofibroblast transdifferentiation by TGF-β1 induced lower levels of decorin and myostatin both in DMD and control cells. These changes are probably related to the existence of co-regulatory interrelationships between TGF-β1, myostatin, and decorin in the fibrotic process (Miura et al. 2006; Li et al. 2008; Zhu et al. 2007).

In vitro, TGF-β1 induces variation in gene expression of MMPs and TIMPs (Roberts and Sporn 1996; Wells 2000). There are also reports on differing effects of TGF-β1 on different MMP isoforms (Uria et al. 1998). We found a reduction in MMP-7 transcript and protein levels in DMD cells after TGF-β1 treatment, but an increase in MMP-1 transcripts and protein. As noted, MMP-1 is the only enzyme able to initiate breakdown of the interstitial collagens type I, II and III. We hypothesize that the increase in MMP-1 in DMD cells might be a compensatory effect of the increase in collagens.

Finally, the significant increase in TIMP-3 transcript and apparent increase in protein levels after TGF-β1 treatment both in DMD and controls is likely to be related to a generic up-regulation of TIMPs by this cytokine (Wells 2000).

To conclude, the study has revealed several differences between fibroblasts from DMD muscle and those from normal muscle, particularly as regards factors likely to affect ECM turnover; these

differences are accentuated by TGF-β1 treatment. These findings confirm that fibroblasts and myofibroblasts play a major role in muscle fibrosis, as also reported for other tissues, and indicate that quantity and localization of both cell populations should be evaluated in in vivo studies. We propose the transdifferentiation of muscle fibroblasts as a simplified model of fibrosis that may be useful for elucidating muscle fibrosis mechanisms occurring at the various steps of ECM deposition, and for testing possible anti-fibrotic agents.

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Chapter 5

Summary, conclusions and future perspectives

DMD is one of the commonest hereditary muscular diseases and is characterized by progressive muscle degeneration in 1 out of 3500 male live births. The gene responsible for DMD is located on the X-chromosome (Koening M et al, 1987); it is the largest and among most complex of known genes, comprising 79 exons and spanning more than 2.4 million base pairs (Koening M et al, 1987). The gene encodes several dystrophin isoforms of which the main muscle isoform is one of the largest proteins. Dystrophin protein is biochemically associated with the membrane-bound dystrophin-glycoprotien complex (DAPC), which forms an important link with laminin, a costituent of the ECM. The DAPC is part of a larger complex of proteins associated with dystrophin, which includes dystroglycan, sarcoglycans, syntrophins, dystrobrevin and utrophin. Mutations in the genes encoding various members of the DAPC disrupt sarcolemmal integrity and result in a variety of muscular dystrophies. DMD patients seem able to compensate for the disease process until around two-five years of age when they show waddling gait and difficulties in running and climbing stairs.

Ongoing cycles of muscle fiber degeneration and regeneration and tissue inflammation, followed by progressive muscle loss and replacement of muscle fibers with fibro-fatty tissue, and ultimately, by muscle wasting with contractures, are the unfortunate consequences of the disease (Emery AEH, 1993). Inflammation and fibrosis are then prominent characteristics of this disease (Morrison J et al, 2000). The timetable of

specific skeletal and cardiac muscle involvement varies significantly from patient to patient, and depends on the differential development over time of inflammatory and fibrotic changes.

The inflammatory reaction and resultant fibrosis are poorly understood but, in general, it is thought to be the sequel of inflammatory infiltrate caused by ongoing muscle necrosis in DMD patients.

Inflammation due to muscle fiber damage is a T-cell mediated process that plays a critical role in transdifferentiation of myofibroblast from resident interstitial fibroblasts and in the activation of satellite cells. The chemotatic factors released from damaged muscle attract leukocytes at the site of injury (Orimo S et al, 1991). Leucocytes produce cytokines that up regulate inflammation and attact macrophages (Best TM and Hunter KD, 2000). Macrophages cause membrane lysis in order to destroy all damged muscle fibers and increse muscle injury (Nguyen HX and Tidball JG, 2003). A second non-phagocytotic population of macrophages invades the muscle (McLennan IS, 1993), produces IL-6, thereby stimulating satellite cell proliferation (Allen RE et al, 1995). The inflammatory process goes on and on in response to the continuous fiber necrosis, but eventually the regeneration process is unable to keep pace with the requirement for new fibers, and massive connective tissue deposition occurs. Fibrosis that results from this inflammatory response plays an important role in the progression of the disease that leads to patient death. Among DMD patients, ultimately 90% of them develop fibrotic changes that result in structural alteration of muscle architecture and progressive loss of function; in cardiac anomalies and respiratory insufficiency; and 53-90% of patients die from respiratory failure (Hunsaker RH et al, 1982; Phillips MF et al, 1999).

Fibrosis is a complex mechanism, often defined as a wound-healing response that has gone out of control. This repair process typically involves two distinct stages: a regenerative phase, where injured cells are replaced by cell of the same type, leaving no lasting evidence of damage; and a phase where connective tissue replaces normal parenchymal tissue. It is now well established that while fibrosis occurs, its pathological hallmarks are quite comparable, irrespective of the tissue affected. These include: enhanced and aberrant activation of profibrotic growth factors; accumulation of activated myofibroblasts; altered composition and increased deposition of ECM; and persistent inflammation, which perpetuates fibrotic transformation (Hewitson TD, 2009).

Although TGF-β1 gene is upregulated in response to tissue injury and though to be the most potent pro fibrotic cytokine, other cytokines, including PDGF, bFGF and IL-1, exert synergistic effect on the progression of fibrosis (Eddy AA, 2000). TGF-β1 is actively involved in connective tissue proliferation in DMD muscle and its maximal production is age related, preceding the severe muscle structural disorganization that occurs later (Bernasconi P et al, 1995). More recently, in our in vitro model, we have detected a significant upregulation of TGF-β1 expression in both myoblast and fibroblast lineage isolated from DMD muscle biopsies (Zanotti S et al, 2007; Zanotti S et al, 2009). TGF-β1 appears to be the most important cytokine for the transdifferentiation of fibroblasts into myofibroblasts, causing the increase of α-SMA expression and the activated phenotype by promoting expression of ECM genes and suppressing the activity of genes such as MMPs. The presence of myofibroblasts has been described in all fibrotic situations characterized by tissue remodelling. Once the tissue damage is

repaired, myofibroblasts are removed by apoptosis; while the persistence of myofibroblast within a fibrotic lesion, as a consequence of permanent tissue damage, leads to excessive scarring with the functional impairment of the affected organ.

During development of the fibrotic process the excessive accumulation of matrix components is the consequence of two parallel processes: increased synthesis and decreased degradation of ECM. The balance between MMPs and TIMPs is largely responsible for the homeostatis of ECM. A deregulation of this balance is a characteristic of diverse pathological conditions such as fibroproliferative diseases including the pulmonary fibrosis, systemic sclerosis, liver chirrosis, cardiovascular disease and progressive kidney disease.

A major advancement towards the understanding of the complexity of the molecular mechanisms that charcterize fibrosis is derived from a consistent number of in vitro studies. Several in vitro studies have been performed especially on pulmonary, liver and skin fibrosis while very few reports have described the cellular mechanims involved in the development of fibrosis in DMD muscle. To better understand the molecular mechanisms involved in muscle fibrosis we have developed an in vitro model based on the immunomagnetic separation of fibrogenic and myogenic lineage from muscle biopsies obtained from DMD patients and age-matched controls.

The major focus of my first part of PhD research project has been the identification of the different contributes of myoblasts and fibroblasts isolated from muscle biopsies, in the ECM remodelling occuring during the fibrotic process. Determining the relative role of these two cell lines in the fibrotic processes will be usefull in the development of new

treatment strategies for contrasting fibrosis. To investigate whether ECM components contribute to the severe fibrotic process observed in DMD muscle, we analyzed at transcript and protein levels, several ECM components in primary DMD and control myotubes. We have shown that the expression of decorin transcripts is significantly reduced while the expression of TGF-β1 transcripts is significantly increased in DMD myotubes compares to controls. Previously, we had observed similar differences in the expression of these molecules in vivo in DMD muscle biopsies (Zanotti S et al, 2005). Transcript expression of the functional ECM components MMP-2, TIMP-1 and TIMP-2 was also significantly increased in DMD myotubes as was the proteolytic activity of MMP-2. The increased expression of MMP-2, TIMP-1 and TIMP-2 in DMD myotube culture medium would probably contribute to alter the equilibrium in ECM homeostatis. In fact TIMPs provide a natural counterbalance to the MMPs activity and disruption of this ordered balance can lead to excess or insufficient matrix degradation. These data suggest that mechanisms regulating ECM turnover in muscle tissue are altered in DMD and that the muscle cells contribute to this process by allowing leakage of cellular components to the extracellular space, or abnormal cellular uptake of extracellular growth factors and cytokines. Furthermore it is possible that dystrophin absence itself could exert a direct influence on ECM hoemostasis.

Another important finding of this study was that both transcript and protein levels of myostatin were significantly higher in DMD myotube cultures than controls. Myostatin is a potent inhibitor of satellite cell activation and cell renewal (McCroskery et al, 2003). Myostatin also binds to decorin (Miura T et al, 2006), a small proteoglycans with

antifibrotic effects (Fukushima K et al, 2001). Our findings that myostatin is increased and that decorin, a potent binder and downregulator of TGF-β1 and myostatin, is decreased may have implications for DMD therapy that aims to reduce muscle fibrosis.

To further characterize the fibrotic process in DMD, we also analyzed the contribution to ECM formation of fibroblasts and myofibroblasts present in muscle cultures. Fibroblasts play a central role in maintaining the integrity of the ECM by synthesising and degrading the ECM components.

The primary goal of the second part of my PhD project was to study the role of primary muscle-derived fibroblasts and of the activated myofibroblasts, in the pro-fibrotic mechanisms of DMD muscle. This study shows that primary fibroblasts derived from DMD muscle biopsies differ fron control fibroblasts in a number of respects. In particular DMD fibroblasts were characterized by a significant increased of both cell proliferation and production of soluble collagens that in control fibroblasts. DMD fibroblasts show a significant increase both in decorin and myostatin transcripts and proteins. Another finding of this study is that MMP-7 transcript and protein levels were increased in DMD fibroblasts. MMP-7 expression is low in the normal liver and is highly upregulated in response to progressive liver fibrosis (Huang CC et al, 2005). A recent study in idiopathic pulmonary fibrosis shows a similar trend suggesting that MMP-7 is a key regulator of pulmonary fibrosis in mice and humans (Zuo F et al, 2002). These data suggest that the expression of MMP-7 may be associated with tissue remodelling during the progression of fibrosis in different organs. We also found a significant reduction in mRNA and protein levels of MMP-1 in DMD

fibroblasts than controls. MMP-1 plays an important role in limiting fibrosis by degrading collagen I and III. The reduction in MMP-1 expression in association with the increased production of soluble collagen and the increase of collagen I expression suggest a fibrotic phenotype of DMD fibroblasts. We also found that DMD fibroblasts have a significant reduction of transcript levels of TIMP-3 than controls. TIMP-3, in addition to its inhibitory action on MMPs, is able to induce apoptosis in various cell types (Ahonen et al, 1998). This finding suggests that DMD fibroblasts may be more resistant to apoptosis that control fibroblasts.

Future perspectives

The major focus of research on therapeutic approaches for DMD has been directed toward alleviating the primary genetic defect through gene therapy or myoblast transfer, both aimed at promoting dystrophin expression in muscle fibers. The most significant obstacles in development of an effective treatment have been both the large size of the dystrophin gene and the fact that it is expresses in all muscles in addition to brain.

Unfortunately for these patients there is currently no cure. Thus the development of complementary and supportive therapies that slow the progression of the disease and allow patients to have an improved quality of life is critically important. Anti-fibrotic therapies may help slow down disease progression and render future more targeted treatments more effective, e.g. by facilitating circulation of drugs or cells within muscles or by favouring tissue regeneration. Delaying and reducing skeletal muscular fibrotic tissue growth should slow the loss of muscle function,

thereby enhancing patient's quality of life, particularly by postponing onset of wheelchair dependence and related adverse consequences suffered by DMD patients. Thus far medical science has been unsuccessful at producing a safe, pharmaceutical treatment to reduce skeletal muscle fibrosis. Conventional therapies for fibrosis using corticosteroids and other anti-inflammatory agents have had little to no clinical efficacy.

In fact the presence of an active fibrogenic process in dystrophic muscles may render therapy for the replacement of dystrophin less feasible and less effective.

Besides the obvious consideration that in vitro data do not fully capture the situation in the whole organ and organism, our in vitro model can be proposed as a simplified model of fibrosis that may be useful for further elucidate molecular mechanisms of muscle fibrosis occurring at the various steps of ECM deposition. Furthermore we can propose this in vitro model as a sensible tool for testing possible anti-fibrotic agents.

Our data show different involvement of myogenic and fibrogenic cell lines isolated from DMD muscle biopsies in ECM remodelling during fibrotic process. Future research should aim at translating our recent knowledge of molecular mechanisms of the fibrotic process in dystrophic muscle into the clinical setting by identifying new drug targets and defining new therapeutic strategies aimed at treating inflammatory and fibrotic disorders.

One possible therapeutic target for treatment of fibrotic disorder is MMP-1. The potential utility of MMP-1 in the treatment of fibrotic conditions derives from its successful use in some organ systems, most notably experimentally induced hepatic fibrosis, where MMP-1 has been shown

to reverse fibrosis and begin the restoration of normal tissue architecture and function (Iimuro Y et al, 2003).

The repair process of injured muscle is a balance between the regeneration of myofibers and the deposition of connective tissue that supports these myofibers. The predominant components of ECM connective and fibrous tissue are collagens I and III. The introduction of MMP-1, which specifically targets collagen I and III, may help in the removal of fibrotic barrier and in facilitating muscle regeneration.

The use of MMP-1 in the treatment of skeletal muscle fibrosis is very recent. In a preliminary study Bedair et al (2007) have shown that the direct injection of high concentration of MMP-1 did not show any negative effect to the muscle fibers nor to the structural integrity of the ECM normally surrounding muscle fibers. Furthermore the MMP-1 delivery into the area of induced fibrosis resulted in the dual therapeutic effect of decreasing residual fibrous tissue and enhancing the repair process. This effect was observed in spite of the short half-life of MMP-1 in vivo. However, the use in this study of the pro-form of MMP-1, made difficult to determine the amount of enzyme that was activated in the scarred tissue prior to it being enzymatically degraded or cleaved via diffusion from the tissue. With the intent to overcome this limitation and to increase the MMP-1's stability, Kaar et al (2008) proposed a more efficient approach based on the use of a more stable active form of MMP-1. Further studies will be necessary in order to obtain more stable MMP-1 isoforms characterized by an enhanced half-life for a better treatment of fibrosis in dystrophic muscles. For improved therapeutic results it will also be important to establish at which phase of the fibrotic process the administration of MMP-1 is most effective.

Another important suggestion emerging from our study regards the potential use of MMP-7 as a non-invasive biomarker of the fibrotic process progression, to be used in the follow-up of muscular dystrophy.

MMP-7 is used as a sensible serum prognostic and predictive marker in different type of tumours (Leelawat K et al, 2009; Acar A et al, 2008) and was also reported to be a key factor determining the prognosis or reversibility of pulmonary (Huh JW et al, 2008), liver (Huang CC et al, 2005) and tubulointerstitial fibrosis (Surendran K et al, 2004). In all these fibrotic conditions, MMP-7 is identified as a major MMP whose expression is increased during the progression of fibrosis.

Furthermore, downregulation of MMP-7 might be able to influence the course of tissue remodelling and change the outcome of the fibrotic process. It is possible therefore to hypothesize that MMP-7 could be used both as a potential therapeutic target in the treatment of fibrosis and as a sensible serum biomarker to follow, in a non-invasive way, the progression of fibrosis.

Finally, as a future perspective, we will continue our studies on elucidation of muscle fibrosis pathogenetic mechanisms by evaluating more ECM components in in vivo patient muscle, both in DMD and in other dystrophic conditions. We will also further investigate, in in vitro studies, the significance of the apoptotic process in fibroblast-myofibroblast persistence or elimination, and the relevance of Wnt/β-catenin pathway. In fact, recent studies suggest that aberrant activation of Wnt signalling has implicated in the pathogenesis of some human fibrosing diseases (Surendran K et al, 2002; Cheon S et al, 2005; Sato M 2006).

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