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Adhesion and Biomechanics in Regenerating Skeletal Muscle

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ACADEMIC DISSERTATION

to be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere, on March 9th, 2001, at 12 o'clock.

Acta Universitatis Tamperensis 796
University of Tampere
Tampere 2001

ACADEMIC DISSERTATION
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http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 796
ISBN 951-44-5036-1
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 87
ISBN 951-44-5037-X
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2001

To Janne, Santeri and Julius

CONTENTS

LIST OF ORIGINAL PAPERS

This thesis is based on the following original publications, referred to as I-IV in the text:

I Kääriäinen M, Kääriäinen J, Järvinen TLN, Sievänen H, Kalimo H, Järvinen M (1998): Correlation between biomechanical and structural changes during the regeneration of skeletal muscle after laceration injury. J Orthop Res 16: 197-206.

II Kääriäinen M, Kääriäinen J, Järvinen TLN, Nissinen L, Heino J, Järvinen M, Kalimo H (2000): Integrin and dystrophin associated adhesion protein complexes during regeneration of shearing-type muscle injury. Neuromusc Disord 10: 121-132.

III Kääriäinen M, Liljamo T, Pelto-Huikko M, Heino J, Järvinen M, Kalimo H (2001): Regulation of α7 integrin by mechanical stress during skeletal muscle regeneration. Neuromusc Disord (in press).

IV Kääriäinen M, Nissinen L, Järvinen M, Heino J, Kalimo H (2001): Expression of α7β1 integrin splicing variants during skeletal muscle regeneration. (submitted for publication)

ABBREVIATIONS

General:

α-DG	α-dystroglycan
α-SG	α-sarcoglycan
ANOVA	analysis of variance
ATP	adenosintriphosphate
β-DG	β-dystroglycan
BEV	backround error value
β-SG	β-sarcoglycan
BM	basement membrane
BMD	Becker muscular dystrophy
bp	base pair
cDNA	complementary DNA
CMD	congenital muscular dystrophy
C-terminal	carboxy terminal
CZ	central zone
DGC	dystrophin glycoprotein complex
DMD	Duchenne muscular dystrophy
ECM	extracellular matrix
ε-SG	ε-sarcoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Grb2	growth factor receptor-bound protein 2
γ-SG	γ-sarcoglycan
H&E	hematoxylin & eosin
IF	intermediate filament
kd	kilodalton
LGMD	limb girdle muscular dystrophy
mRNA	messenger ribonucleic acid
MTJ	myotendinous junction
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
N-terminal	amino-terminal
PH	pleckstrin homology
ROD	relative optical density
RT-PCR	reverse transcriptase-polymerase chain reaction
RZ	regeneration zone
δ-SG	δ-sarcoglycan
SSC	standard saline citrate
SZ	survival zone

Rat groups in study III:

FM	Free mobilization
MO	Mobilization
IM	Immobilization
DE	Denervation
IM+DE	Immobilization + Denervation

INTRODUCTION

Skeletal muscle is capable of extensive regeneration after injury. Satellite cells, located between the basement membrane (BM) and plasma membrane of the myofiber, are responsible for the regeneration process (Mauro 1961, Schultz 1989, Grounds 1991). They are quiescent myogenic precursor cells, which become activated following disruption of the sarcolemma and muscle necrosis in muscle injury. Satellite cells proliferate and differentiate into myoblasts. Myoblasts then fuse into multinucleated myotubes and begin to produce muscle-specific proteins and finally mature into adult muscle fibers. The regeneration process recapitulates myogenesis during fetal development and is similar irrespective of the mechanism of the injury (Allbrook 1981, Bodine-Fowler 1994).

Muscle injuries can be classified into shearing and in situ necrosis type of muscle injury. In shearing injury, which is the more common type of injury, not only the myofibers but also their BM and the mysial sheaths are torn and thus the functional continuity of the muscle-tendon complex is disrupted. In the in situ necrosis type of muscle injury myofibers are necrotized within their intact BM. In shearing injury, during the repair process the stumps of the ruptured regenerating myofibers must repair their structural integrity and bind firmly to the extracellular matrix (ECM) to re-establish the functional continuity. Restoration of the connection between the contractile proteins of the regenerating myofibers and ECM makes it possible to transform the force of myofiber contraction into movement allowing the use of the muscle. The purpose of this work was to study structural changes and the re-establisment of the adhesion between the regenerating myofibers and ECM during muscle regeneration, as well as the effect of altered mechanical stress on the adhesion process and to correlate these changes with the restoration of tensile strength.

REVIEW OF THE LITERATURE

1. Structure of skeletal muscle

1.1. Myofiber

Skeletal muscle is composed of muscle cells, usually called myofibers (Fig. 1), which are multinucleated and elongated cells, 10 to 100 μm in diameter and from a few millimeters to several centimeters long. Most of the myofiber nuclei seen in the light microscope are located peripherally beneath the plasma membrane. About 5 percent of all the myonuclei at the periphery of myofibers belong to the satellite cells, which are located between the plasma membrane and BM. BM surrounds the myofiber separating it from the ECM. (Ross and Romrell 1989, Landon 1992)

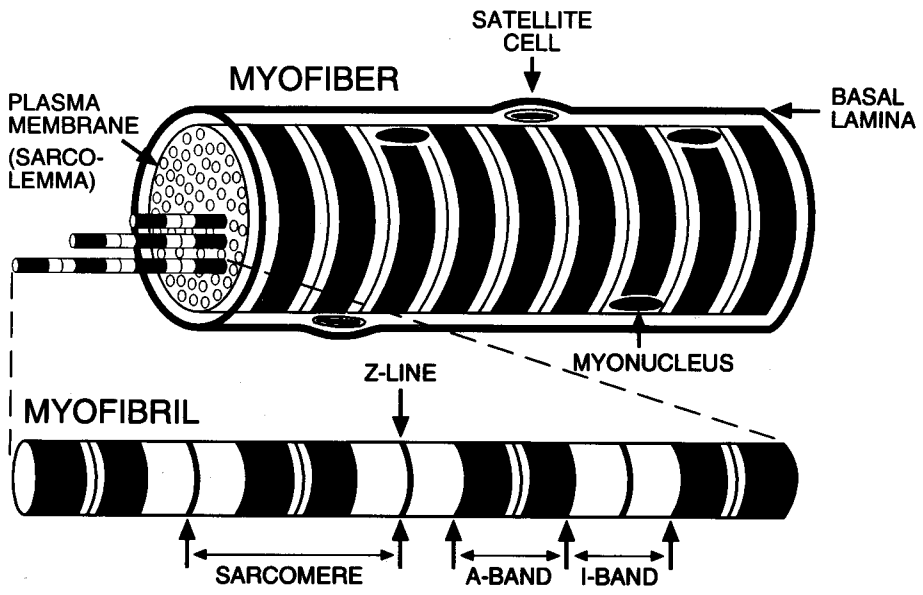

Fig. 1. Structure of the myofiber.

The main structural components of the sarcoplasm in each myofiber are bundles of myofilaments called myofibrils (Ross and Romrell 1989, Landon 1992) (Fig. 1). Sarcomeres, which are serially linked in rows, form the contractile units of the myofibrils. Sarcomeres contain muscle specific proteins, mostly thin actin and thick myosin filaments. Regular organization of these filaments in sarcomeres creates the

typical cross-striated appearance of skeletal muscle. Sarcomeres are delineated at their ends to the Z-discs, whereby actin filaments are attached. α-actinin is a structural protein abundant in the Z-lines and may link thin filaments at the Z-discs. Thick filaments are also linked to the Z-discs by elastic filaments made of titin (Patel and Lieber 1997). The A-I band junctions in the myofibrils are surrounded by T tubules, which are an extension of the plasma membrane forming junctions with the sarcoplasmic reticulum and which function in the conduction of muscle exitation. Actin filaments form a lighter I-band, which is transected by the Z-line. Myosin filaments in the middle of sarcomere form the darker A-band. Actin filaments also extend partially into the A band, where they interdigitate with the myosin filaments. Thin filaments of the I-band also contain nebulin, tropomyosin and troponin molecules. The sliding mechanism of the filaments is the basis for muscle contraction. During contraction filaments interact so that thin filaments move past the thick filaments toward the center of the sarcomere shortening the sarcomere. (Ross and Romrell 1989, Landon 1992)

The cytoskeleton of myofibers contains intermediate filaments (IFs) with a diameter of approximately 10 nm (intermediate between actin and myosin) (Lazarides 1980). Vimentin (Granger and Lazarides 1979), desmin (Lazarides and Hubbard 1976) and nestin (Lendahl et al. 1990, Sejersen and Lendahl 1993) are expressed in skeletal muscles. IFs may have an important role in cellular organization during myogenesis and in maintaining structural integrity in mature myofibers. Vimentin and nestin are expressed during early developmental stages of the prenatal period, whereas desmin expression is initiated at later stages (Sejersen and Lendahl 1993, Vaittinen et al. 1999). In mature myofibers vimentin expression is completely down-regulated whereas nestin is expressed at low levels adjacent to neuromuscular junctions (NMJ) and myotendinous junctions (MTJ). Desmin expression increases continuously with advancing maturation and it accumulates finally at the margins of Z-discs anchoring the Z-discs of adjacent myofibrils together and interconnecting myofibrils to the plasma membrane (Lazarides 1980, Tokuyasu et al. 1985).

The BM, with a thickness of 30 to 120 nm, forms an extracellular layer surrounding the myofiber (Timpl and Dziadek 1986). BM is important in maintaining structural integrity and has functions in cell adhesion and signaling (see below) (Martinez-Hernandez and

Amenta 1983, Engvall 1993). It is mainly secreted by the muscle cell itself but fibroblasts may also contribute some of its components (Kühl et al. 1982, Abrahamson 1986). BM consists of two layers: the inner *lamina rara (lamina lucida)* and outer *lamina densa*. Type IV collagen forms a basal network in BM, where laminin, fibronectin and heparan sulphate proteoglycans are embedded (Martin and Timpl 1987). A reticular layer of endomysium consisting of collagen fibrils (e.g. type III collagen) lines BM externally (Abrahamson 1986).

1.2. Connective tissue in muscle

Myofibers are held together by connective tissue, which is organized into three distinct sheaths (Borg and Caulfield 1980). Endomysium is a delicate, loose connective tissue surrounding each myofiber and containing the capillaries. BM is included in the endomysium. Perimysium surrounds bundles of myofibers or fascicles and serves as a route for the larger blood vessels. Epimysium is relatively dense connective tissue surrounding the whole muscle belly.

Collagens are the most abundant molecules in muscle ECM. They provide strength and stability for the tissue framework and play a role in the growth and differentation processes (Mayne and Sanderson 1985). Type I collagen fibers are thick and strong and are mainly found in epimysium and perimysium. Type III collagen fibers are thinner and more flexible and are distributed in all the collagenous structures of muscle being extensively present in the endomysium and perimysium (Duance et al. 1977, Lehto et al. 1985b). Type IV collagen is confined to the endomysial BM structures only, with a codistribution of type V collagen (Duance et al. 1977, Lehto 1983). Minor amounts of type V collagen are also present in the perimysium.

1.3. Innervation and neuromuscular junctions

Each myofiber is innervated by an axon terminal and they respond to impulses conducted by motor neurons of the spinal cord or brain stem. The contact site between the terminal branches of the axon and muscle is called the motor end plate or neuromuscular junction (NMJ). Plasma membrane and BM form a folded structure in the NMJ increasing the receptor area in myofiber. A single motor neuron may contact some tens to over one thousand myofibers, but each myofiber is innervated by one

nerve cell and one axon terminal only. NMJ is usually located in the middle third of the myofiber. Nerve cells not only serve to instruct the muscle cells to contract but also exert a trophic influence on the muscle cells, which is necessary to maintain the structural integrity of muscle cells. (Ross and Romrell 1989, Landon 1992)

2. Interaction between myofibers and ECM

Interaction between the structural proteins of ECM and cytoskeletal proteins is necessary to transform the force generated by contractile proteins into movement (Tidball 1991, Law and Tidball 1993). Connection between myofibers and ECM also provides stability for the sarcolemma and a route for the transmission of mechanical and chemical signals from the surrounding ECM to the cell and vice versa. The adhesion of myofibers to the surrounding ECM is accomplished by two main complexes of adhesion molecules, integrin and dystrophin associated (Hynes 1987, Ervasti and Campbell 1993a, Sunada and Campbell 1995, Brown 1996, Meredith et al. 1996, Giancotti and Ruoslahti 1999) (Fig 2.).

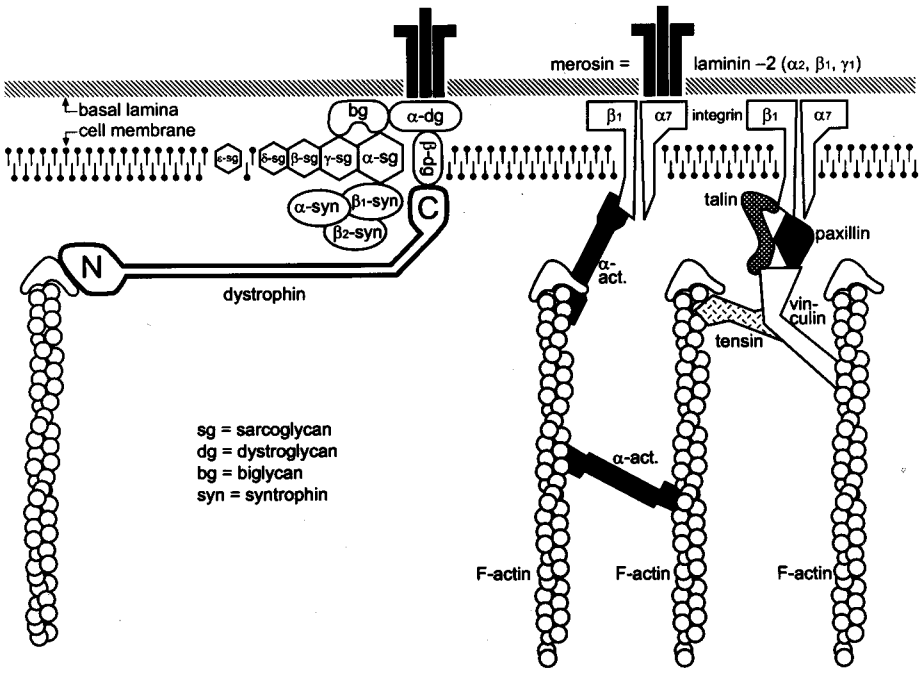

Figure 2. Molecules involved in the adhesion of myofibers to the extracellular matrix.

2.1. Adhesion molecules

2.1.1. Integrins

Integrins are transmembrane cell adhesion molecules that participate in the complex biological process of embryonic development and in the maintenance of tissue integrity (Hynes 1987, Meredith et al. 1996, de Melker and Sonnenberg 1999, Giancotti and Ruoslahti 1999). They also function in wound healing and pathological processes such as inflammation and malignant transformation by affecting cellular activities like cell growth, differentiation, migration, spreading, and apoptosis. Integrins consist of two subunits, α and β, which contain both extracellular and cytoplasmic domains (Hynes 1992). Seventeen different α and eight β subunits have been identified in vertebrates (de Melker and Sonnenberg 1999). In addition, alternative splicing of integrin messenger RNA leads to variations in the sequence of both extracellular and cytoplasmic domains of several α and β subunits. β subunits are necessary for targeting integrins on sites of cell-matrix adhesion, while α subunits mostly determine the specificity of the ligand binding (Hayashi et al. 1990, LaFlamme et al. 1992, Ylänne et al. 1993).

2.1.1.1. β1 integrin

β1 integrin subunit is widely expressed in all adhesion-dependent cell types in different tissues, including both developing and normal adult muscle (Bozyczko et al. 1989, de Strooper et al. 1989, Hynes 1992, Heub and Neundorfer 1997). At least fibronectin, collagens and laminins are extracellular ligands of β1 integrin. β1 integrin has five isoforms with alternatively spliced cytoplasmic domain, β1A, B, C, C-2 and D (Argraves et al. 1987, Altruda et al. 1990, Languino and Ruoslahti 1992, van der Flier et al. 1995, Zhidkova et al. 1995, Belkin et al. 1996, Svineng et al. 1998).

β1A is widely expressed in many tissues and is found in large amounts in proliferating myoblasts during myodifferentiation (Belkin et al. 1996, van der Flier et al. 1997). Later, after myoblast fusion, β1A is gradually replaced by β1D and is expressed only at the low level or hardly at all in adult striated muscle. β1D is a muscle-specific variant of β1 integrin and a predominant β1 isoform in striated muscle (van der Flier et al. 1995, Zhidkova et al. 1995, Belkin et al. 1996). β1D is upregulated during myoblast differentiation and is concentrated in costameres, MTJs and NMJs in mature muscle

(van der Flier et al. 1995, Belkin et al. 1996, van der Flier et al. 1997). The onset of β1D expression during myodifferentiation coincides with the timing of myoblast withdrawal from the cell cycle (Belkin et al. 1996). β1D has also been shown to have growth inhibitory properties. These data suggest an important role for the β1D in the commitment of myoblasts to differentiate into skeletal muscle cells (Belkin and Retta 1998). Alternative splicing can also serve as a physiological mechanism to modulate adhesive function. The affinity of the interaction between cytoskeleton and β1A and β1D isoforms is different. β1D integrin has stronger interaction with cytoskeletal actin than β1A integrin (Belkin et al. 1997). Thus, β1D seems to have an important role in forming the extremely stable cytoskeleton-ECM association required for contraction in mature muscle.

β1B and β1C are minor isoforms, which have restricted tissue distribution and are unable to localize to cell-matrix adhesion sites apparently because of impaired interaction with the actin cytoskeleton (Altruda et al. 1990, Languino and Ruoslahti 1992, Balzac et al. 1993, Meredith et al. 1995). β1B has been speculated to serve as a negative regulator of cell adhesion during development, whereas β1C can strongly inhibit cell growth (Balzac et al. 1993, Balzac et al. 1994, Meredith et al. 1995). Recently a new splice variant β1C-2 has been identified (Svineng et al. 1998).

2.1.1.2. α7 integrin

β1 integrin subunit associates with several α subunits to form distinct integrin dimers, capable of interacting with various ECM molecules as well as other cell adhesion molecules and capable of transducing signals across the plasma membrane (Hynes 1992). α7β1 is the predominant integrin on skeletal muscle that binds merosin (von der Mark et al. 1991, Song et al. 1992, Hodges and Kaufman 1996, Burkin and Kaufman 1999). α7 integrin has three isoforms with alternatively spliced cytoplasmic domains (α7A, B and C) (Collo et al. 1993, Song et al. 1993, Ziober et al. 1993) and two isoforms with alternatively spliced extracellular domains (α7X1 and X2) (Ziober et al. 1993, Hodges and Kaufman 1996). Alternative splicing produces six variants of the α7 integrin chain because each cytoplasmic isoform can contain either X1 or X2 extracellular domain. β1 associated α8 integrin has been detected in smooth muscle

(Schnapp et al. 1995). Tenascin-C, which is a major ECM protein in tendon, is one of its ligands (Varnum-Finney et al. 1995). Thus, it seems likely that α8 integrin also has a role in myofiber-ECM adhesion in skeletal muscle.

Alternatively spliced cytoplasmic domains of α7A, α7B and α7C differ in size, in sequence and in their potential for phosphorylation, which has an effect on the signal-transducing capacity (Collo et al. 1993, Song et al. 1993, Ziober et al. 1993). The cytoplasmic domain of the α7B isoform is particularly potential in participating in the transduction of signals. α7B is actively expressed in proliferating, mobile myoblasts but its level of expression diminishes upon differentiation though it also persists in adult myofibers. α7B is also expressed in other tissues in contrast to α7A and α7C isoforms, which are detected only in skeletal muscle. α7A and α7C isoforms are expressed upon terminal myogenic differentation, coincidentally with the expression of myogenin (Song et al. 1993, Martin et. al. 1996). The switch from one α7 integrin isoform to another may be an important mechanism in skeletal muscle differentiation. Regulatory mechanisms could be associated with ligand binding, cytoskeletal interactions, and signal transduction.

α7X1 and α7X2 isoforms with alternatively spliced extracellular domains are both found in myogenic cells, X2 being the predominant variant (Ziober et al. 1993, Hodges and Kaufman 1996). The ratio of X1/X2 transcripts does not vary significantly in replicating myoblasts and myotubes (Hodges and Kaufman 1996). Adult skeletal muscle expresses only X2 isoforms whereas other cell types express both isoforms. The two isoforms have functional differences (Crawley et al. 1997, Ziober et al. 1997). α7X1 appears to have higher binding affinity to a mixture of laminin-2 (i.e. merosin) and laminin-4 than to laminin-1, whereas α7X2 binds equally well to each laminin isoform (Crawley et al. 1997). It has been proposed that X1 isoform is important during dynamic adhesion related to muscle development (motility, fusion, remodeling, repair and matrix assembly) and that the X2 variant performs more stable adhesion functions (costameres and MTJs) (Ziober et al. 1997).

In the integrin associated molecule complex, sarcomeric actin binds via several subsarcolemmally located molecules, such as α-actinin, talin, vinculin, paxillin and tensin to the cytoplasmic domain of β1 integrin (Horwitz et al. 1986, Otey et al. 1990, Otto 1990, Jockusch et al. 1995, Meredith et al. 1996). Integrin α7β1 in turn binds via its α7 subunit to its extracellular ligand merosin, forming a connection between the cytoskeleton and ECM (von der Mark et al. 1991, Yao et al. 1996). Integrin α7β1 is enriched in the MTJs and NMJs with only minor amounts being present on the lateral aspects of the myofiber plasma membrane (Bao et al. 1993, Martin et al. 1996).

Analysis of muscle biopsies from previously undefined congenital myopathy patients have revealed few α7 chain deficiencies (Hayashi et al. 1998). These patients showed mutations in the integrin α7 gene. α7 integrin null mice develop a muscular dystrophy with histopathological changes indicating impairment of function of MTJs (Mayer et al. 1997). α7β1 integrin expression is also altered in other muscular dystrophies (Hodges et al. 1997). α7β1 integrin and dystrophin mediated transmembrane linkages between the ECM and cytoskeleton may have some overlap or synergistic functions. Enhanced expression of α7β1 integrin has been found in disease entities with dystrophin deficiency, *i.e*. in the muscle of *mdx* mouse and in patients with Duchenne or Becker muscular dystrophy (DMD and BMD) (Hodges et al. 1997). In addition, the localization of α7A and α7B in *mdx* mouse was found to expand to the extrajunctional regions of muscle fibers from the junctional sites (Burkin and Kaufman 1999). In contrast, patients with laminin α2 chain congenital muscular dystrophy or *dy/dy* mice lacking α2 laminin chain have decreased level of α7β1 integrin in muscle (Hodges et al. 1997, Vachon et al. 1997). It has been suggested that laminin-2 (i.e. merosin) may regulate the expression of the α7 integrin gene (Hodges et al. 1997).

2.1.2. Dystrophin glycoprotein complex

The search for the defective molecule in DMD in 1987 led to the discovery of dystrophin (Hoffman et al. 1987, Koenig et al. 1988, Sadoulet-Puccio and Kunkel 1996) and dystrophin associated proteins (Campbell and Kahl 1989, Worton 1995, Brown 1996). Dystrophin is a large subsarcolemmally located protein (Minetti et al. 1992, Porter et al. 1992), which is expressed both in the smooth and striated muscle (both

skeletal and cardiac) and in minor amounts in nerve tissue (Hoffman et al. 1988, Sadoulet-Puccio and Kunkel 1996). Dystrophin is enriched in MTJs and NMJs in skeletal muscle (Samitt and Bonilla 1990, Byers et al. 1991). Dystrophin is composed of four domains: an N-terminal actin-binding, a large rod-shaped spectrin-like, a cysteine-rich and a C-terminal domain (Hammond 1987, Koenig et al. 1988). Dystrophin represents only about 0.002% of the total skeletal muscle protein (Hoffman et al. 1987), but constitutes approximately 5% of the total membrane cytoskeleton fraction of skeletal muscle sarcolemma (Ohlendieck and Campbell 1991), i.e. dystrophin is a major component of the subsarcolemmal cytoskeleton in skeletal muscle.

Dystrophin forms a dystrophin glycoprotein complex (DGC) with at least three protein complexes: dystroglycans, sarcoglycans and syntrophins (Campbell and Kahl 1989, Ervasti et al. 1990, Yoshida and Ozawa 1990, Ervasti and Campbell 1991, Yoshida et al. 1994). Dystroglycan complex consists of transmembrane β-dystroglycan (β-DG) (43 kd) and extracellular α-dystroglycan (α-DG) (156 kd), which are encoded by a single mRNA (Ibraghimov-Beskrovnaya et al. 1992). They are expressed both in the muscle and other tissues. Sarcoglycan complex is composed of four transmembrane proteins: α-sarcoglycan (α-SG i.e. adhalin) (50 kd), β-sarcoglycan (β-SG) (43 kd), γ-sarcoglycan (γ-SG) (35 kd) and δ-sarcoglycan (δ-SG) (35 kd) (Ervasti and Campbell 1991, Roberds et al. 1993). α- and γ-SGs are specific for striated muscle (Yamamoto et al. 1994). Recently, a fifth sarcoglycan, ε-sarcoglycan (ε-SG) (25 kd) has also been described (Ettinger et al. 1997, McNally et al. 1998). It is homologous to α-SG and is complexed with other sarcoglycans (Ettinger et al. 1997, Liu and Engvall 1999). Syntrophin complex consists of three cytoplasmic syntrophin proteins (about 60 kd) (Adams et al. 1993, Brown 1996). α1-syntrophin is expressed solely in skeletal muscle while β1- and β2-syntrophins have wider tissue distribution. Another dystrophin related protein dystrobrevin has also been shown to be associated with dystrophin as a component of the DGC (Blake et al. 1996b, Nawrotzki et al. 1998). Dystrobrevin immunoreactivity is restricted to the NMJ and sarcolemma in skeletal muscle. Recently a new member of the DGC, named sarcospan (25 kd), has been identified (Crosbie et al. 1997). Sarcospan is localized in sarcolemma and is associated with the sarcoglycan subcomplex (Crosbie et al. 1999). In addition, α-DG binding polypeptide biglycan has been found (Bowe et al.

2000). Biglycan is localized on the muscle cell surface both at synaptic and nonsynaptic regions.

In the DGC, actin binds to subsarcolemmally located dystrophin (Ervasti and Campbell 1993b, Fabbrizio et al. 1993), which in turn binds with its C-terminus to the transmembrane β-DG (Suzuki et al. 1994, Wakayama et al. 1995). β-DG is linked to the extracellular α-DG (Yoshida et al. 1994), which binds to extracellular merosin in the BM (Ibraghimov-Beskrovnaya et al. 1992, Sunada et al. 1994). Merosin is a muscle specific member of the laminin family and is also present in peripheral nerve (Engvall et al. 1990, Engvall 1993, Tryggvason 1993). Laminins are the major protein component of BMs. Extracellular biglycan binds to α-DG providing an alternative pathway for DGC association with the ECM (Bowe et al. 2000). Dystrophin also binds to the subsarcolemmal syntrophin complex (Ahn and Kunkel 1995, Suzuki et al. 1995, Yang et al. 1995b). Sarcoglycan complex seems to be associated with dystrophin (Yoshida and Ozawa 1990), but binding sites to dystrophin have not been found. It is possible, that the sarcoglycan complex binds to dystrophin via the dystroglycan complex (Yoshida et al. 1994). The exact function of syntrophin and sarcoglycan complexes is unknown, but they may be involved in signaling processes and in maintaining the structural integrity of sarcolemma (see below).

Dystrophin and associated proteins are sequentially expressed during human fetal muscle development (Tomé et al. 1994, Mora et al. 1996). Dystrophin-related protein utrophin is expressed in developing myofibers both at the sarcolemma and at NMJs and MTJs similar to dystrophin, whereas in mature myofibers utrophin is present only at NMJs and MTJs in contrast to dystrophin, which is also present at the sarcolemma (Blake et al. 1996a). Utrophin expression at the sarcolemma gradually declines and is finally replaced by dystrophin expression (Tomé et al. 1994, Mora et al. 1996) syntrophin expressed simultaneously with dystrophin (Tomé et al. 1994). The expression of these cytoplasmic proteins is followed by that of the transmembrane and extracellular proteins of the DGC (Tomé et al. 1994, Mora et al. 1996). α- and β-DG seem to appear in the sarcolemma earlier than α-SG. The production of extracellular merosin by the myofiber in rat is induced around birth after which its synthesis is increased (Engvall 1993). The sequence of protein expression suggests that preceding

expression of subsarcolemmal proteins may have an essential role in the assembly of transmembrane and extracellular components of the DGC during muscle development (Tomé et al. 1994).

Genetic mutations of individual proteins of DGC are manifested as muscular dystrophies (Worton 1995, Brown and Phil 1997, Straub and Campbell 1997). Mutations in dystrophin gene can lead to lack of dystrophin causing Duchenne muscular dystrophy (DMD) or an altered form or reduced amount of dystrophin causing milder Becker muscular dystrophy (BMD). Mutations in sarcoglycan genes cause different types of autosomally recessively inherited limb-girdle muscular dystrophies (LGMD) and mutation in the gene encoding the α2 chain of laminin-2 (i.e. merosin) causes one form of congenital muscular dystrophies (CMD). A common pathogenic feature of all these muscular dystrophies is considered to be the interruption of the link between the ECM and the cytoskeleton, which leads to instability of the sarcolemma. The altered mechanical status of the muscle cell surface caused by the absence of dystrophin has been shown to bring about a long-term accumulation of effects such as modified state of ion channels (Franco-Obregon and Lansman 1994), abnormal fluxes of substances caused by mechanical defect in sarcolemma (McNeil and Steinhardt 1997), and a change in mechanotransduction of growth signals (Brown and Lucy 1993), which ultimately lead to muscle degeneration and cell death. Mechanisms (nonmechanical) other than actual defects in sarcolemma may also be responsible for muscle enzyme release from and deleterious calcium entry into the dystrophic myofiber (Hack et al. 1999).

2.2. Signaling

2.2.1. Integrins

Integrins can signal through the cell membrane in either direction (reviewed in Hynes 1992, Meredith et al. 1996, Chiquet 1999, Giancotti and Ruoslahti 1999). The extracellular binding activity is regulated from the inside of the cell (inside-out signaling), while the binding to the ECM elicits signals that are transmitted into the cell (outside-in signaling). Cytoplasmic tails of integrins are short and devoid of enzymatic features. Hence, integrins transduce signals by associating with adapter proteins. During cell adhesion, the integrin binding to their ECM ligands promotes their clustering and

association with the cytoskeleton with simultaneous aggregation of integrin-associated structural and signaling proteins. Mechanical and chemical signals regulate the activities of cytoplasmic kinases, growth factor receptors, and ion channels and control the organization of the intracellular actin cytoskeleton. Many signals regulate cell cycle, directing cells to live or die, to proliferate, or to exit the cell cycle and differentiate. In addition, integrins may trigger signals after the event of ligand binding, the mechanism of which is essential in mechanotransducing activity (see below).

ECM is the substrate to which cells adhere and on which they grow, migrate and differentiate. ECM also has an important mechanical function. Without it organisms could not maintain their shape, they would be unable to move and would disintegrate. It is likely that the amount and composition of ECM are controlled not only by endogenous cellular programs and growth factors, but also by the kind and magnitude of mechanical stress acting on a tissue (reviewed in Chiquet 1999). Integrins may be key players in transducing signals leading to adaptive cellular responses, which adjust the properties of the ECM appropriate for altered mechanical stress. It has been demonstrated that already existing integrin-mediated contacts are able to sense changes in the local force applied to them, and that they trigger a local increase in the cellular stiffness and traction at this site. It is also possible that stretch responses may be mediated by other signaling molecules associated with integrins at ECM contacts. Alternatively, integrins may be linked to stretch-sensitive ion channels. Integrins and associated proteins can activate multiple signaling pathways in response to mechanical stress. These signaling mechanisms may regulate various cellular functions. There is some experimental evidence showing that, for example, production of ECM proteins tenascin-C and collagen XII is regulated at the level of gene transcription in response to mechanical stimuli in vivo (reviewed in Chiquet 1999). Mechanical forces may also be transduced directly via mechanosensory ion channels, which interact with different proteins of ECM (Liu et al. 1996).

Alternative splicing is a mechanism to regulate the ligand binding and signaling activity of integrins (reviewed in de Melker and Sonnenberg 1999). The expression of splice variants is often tissue specific and/or developmentally regulated. Alternative protein sequences in the extracellular domains provide different ligand-binding sites, which

could result in different specificities for ligand as well as different intracellular signals. Different conformations in extracellular domains may also affect the activation states of integrins and thereby their ligand-binding affinities. For example, the ligand affinity of α7β1 integrin can be regulated by alternative splicing (described above). In addition, heterodimerization of the α and β subunits may be affected regulating the level at which integrins are expressed at the cell surface. Alternatively spliced cytoplasmic domains may activate different signaling pathways and thus result in different cellular responses to ligand binding. Differences in the signaling activity between integrin variants may result from a difference in their ability to bind intracellular signaling molecules.

2.2.2. Dystrophin glycoprotein complex

In addition to connecting ECM with cytoskeleton, DGC may also be involved in signaling, which helps to protect muscles from mechanical injury. For example, β-DG has been shown to interact with Grb2, an adapter protein involved in signal transduction and cytoskeletal organization (Yang et al. 1995a). Syntrophins contain pleckstrin homology (PH) domains (Gibson et al. 1994), one of them being PDZ protein binding domain, which recruit kinases, sodium channels and neuronal nitric oxide synthase (nNOS) to the DGC. Both dystrophin and syntrophins have been shown to be associated with a signaling enzyme nNOS, which is localized to the sarcolemma of fast-twitch fibers (Brenman et al. 1995, Chang et al. 1996, Wakayama et al. 1997). nNOS synthesizes nitric oxide (NO) in skeletal muscle. Synthesis of NO in active muscle opposes contractile force. Interaction between syntrophins and sodium channels has been shown, which suggests that syntrophins link these channels on the one hand to the actin cytoskeleton and on the other hand to ECM via DGC (Gee et al. 1998).

Muscles of mice lacking γ-SG have shown normal resistance to mechanical strain induced by eccentric muscle contraction (Hack et al. 1999). The absence of mechanical deficit in these muscles suggests that the dystrophin-dystroglycan-laminin link is present and functional and thus mechanical weakness and contraction-induced muscle injury are not required for muscle degeneration and the dystrophic process. Thus, γ-SG is likely to have some unknown signaling function, which may be defective, causing muscular dystrophy in γ-SG deficient muscles. This nonmechanical mechanism may also be involved in the pathogenesis of DMD as dystrophin mutations produce a

secondary reduction in sarcoglycan subunits. There is some evidence for a signaling role of α-SG, which has been shown to have an ecto-ATPase activity (Betto et al. 1999). Ecto-ATPases are transmembrane enzymes that catalyze the hydrolysis of extracellular ATP. α-SG may modulate the activity of P2X receptors (a nonspecific cationic channels) by buffering the extracellular ATP concentration. Absence of α-SG leaves the concentration of extracellular ATP elevated and causes persistent activation of P2X receptors, which may lead to intracellular Ca^{2+} overload and muscle fiber death.

2.3. Force transmission

Endo- and exosarcomeric cytoskeletal proteins create series and parallel connections between contractile proteins within the cell resulting in a meshwork across which force can be transmitted in practically any direction with respect to the fiber axis (Patel and Lieber 1997). Connections between the contractile proteins and specialized membrane complexes at the surface membrane provide the route of force transmission to the ECM (Patel and Lieber 1997). Finally, parallel and series connections between myofibers allow radial and longitudinal forces to converge on the connective tissue matrix (Patel and Lieber 1997).

The major force transmission sites are specialized structures called myotendinous junctions (MTJ) located at the ends of myofibers (Tidball 1991, Huijing 1999). The folded structure of the cell membrane at the MTJ significantly reduces the absolute value of stress applied to the cell membrane, ensuring that the principle stress vector at the cell membrane is rather shear than tension (Tidball 1991, Trotter 1993, Huijing 1999). Proteins involved in mediating thin filament-membrane association at sites of cell adhesion to extracellular structural proteins are enriched at the MTJs providing further support for a force transmission role of MTJ. At least fibronectin, collagen and chains of integrin and dystrophin associated molecules may take part in force transmission (Tidball 1991, Law and Tidball 1993).

In many muscles myofibers commonly end within muscle fascicles without reaching a MTJ and many of these fibers show a progressive decline in the cross-sectional area along the length of the muscle. If all of the forces are transmitted via sarcomeres arranged in series, those few sarcomeres at the smaller ends of the fibers must tolerate

the stress exerted by the more numerous sarcomeres arranged in parallel at the portions of the fiber with larger cross-sectional areas (Monti et al. 1999). Therefore, it seems logical that force could also be transmitted laterally along the length of the fiber to the cell membrane and ECM. Such an arrangement would minimize the necessity for a precise level of force to be generated along the entire length of the fiber. The exact mechanisms and structures involved in the process of lateral transmission are very poorly known (Huijing 1999, Monti et al. 1999). Lateral force transmission further supports the idea that tension transmission is a general property of muscle cell surfaces, and that specific junctional morphologies are the results of dynamic interactions between myofibers and the tissues to which they adhere (Trotter 1993).

Subsarcolemmal domains called costameres are believed to be involved in the lateral force transmission across the cell membrane (Tidball 1991). Costameres have similar molecular composition to MTJs. These structures attach cytoskeleton laterally to the cell membrane and are distributed at intervals along the lateral surfaces of myofibers with a periodicity identical to that of Z-discs. In addition, physiological observations showing that damaged myofibers are capable of transmitting force from myofibrils laterally to the cell membrane and ECM give further support a possible role of costameres in force transmission (Street 1983).

3. Regeneration of skeletal muscle

Muscle cells have an extensive capacity to regenerate (Mauro 1961). The regeneration process resembles myogenesis during fetal development and is similar irrespective of the mechanism of the injury (Allbrook 1981, Bodine-Fowler 1994). The most common type of muscle injury is a shearing type of muscle injury, in which not only the myofibers but also their BM and the mysial sheaths are torn in contrast to the in situ necrosis type of muscle injury, in which only the myofibers are necrotized within their intact BM (Kalimo et al. 1997). In shearing injury the functional continuity of the muscle-tendon complex is disrupted. Therefore, during the repair process the proximal and distal stumps of the ruptured myofibers must recover their structural integrity and the regenerating myofibers must also bind firmly to the extracellular matrix (ECM) to re-establish the functional continuity.

3.1. Destruction phase

After muscle shearing injury the ruptured myofibers contract and a gap is formed between them (Hurme et al. 1991a, Kalimo et al. 1997). Because skeletal muscle is richly vascularized, hamorrhage from the torn vessels is inescapable and the gap is filled with a hematoma to be later replaced by scar tissue. Muscle injuries may also be complicated by destruction of intramuscular nerve branches, which will leave the whole or parts of the muscle denervated (Rantanen et al. 1995b, Kalimo et al. 1997). Destruction of the plasma membrane causes extracellular calcium to flow into the cell causing activation of many degrading enzymes, such as phospholipases and neutral proteases (Carpenter and Karpati 1989). Myofibers become necrotized from the site of rupture over a distance of 1-2 mm inside their preserved BM cylinders. The spread of necrosis is limited within hours after injury by a so-called contraction band, i.e. condensation of cytoskeletal material (Carpenter and Karpati 1989, Hurme et al. 1991a). This band forms a barrier that allows the formation of a demarcation membrane, which delineates the regeneration zone from the survival zone, where myofibers survive with certain reactive changes (Carpenter and Karpati 1989, Papadimitriou et al. 1990).

Inflammatory cells gain immediate access to the injury site as the blood vessels are also torn in muscle shearing injury (Tidball 1995). Later, substances released from the necrotized parts of myofibers serve as chemoattractants for further extravasation of inflammatory cells. Macrophages and fibroblasts within the injured muscle are also activated and provide additional chemotactic signals (e.g. growth factors) to further recruit circulating inflammatory cells (Cantini and Carraro 1995, Tidball 1995). Polymorphonuclear leukocytes predominate in the acute phase but are soon followed by monocytes which are transformed into macrophages actively engaged in proteolysis and phagocytosis of the necrotic material (Hurme and Kalimo 1992a, Tidball 1995).

3.2. Repair and remodelling phase

Satellite cells, which are quiescent myogenic precursor cells located underneath the BM of each individual myofiber, are activated during the regeneration process (Mauro 1961, Grounds 1991, Schultz 1989). There are two different populations of satellite cells: committed satellite cells, which are ready for immediate differentiation without preceding cell division, and stem satellite cells, which undergo mitosis beginning

around 24 hours after the injury before providing one daughter cell for differentiation and another for future proliferation (Rantanen et al. 1995a). The exact factors initiating satellite cell activation are poorly known. Extracts from the injured muscle fibers, factors released by invading macrophages and soluble factors from extravasated plasma have been proposed to be involved in the process (Bischoff 1986, Grounds 1991, Hurme and Kalimo 1992a). Activated satellite cells differentiate to myoblasts and fuse with each other into multinucleated myotubes (Carlson and Faulkner 1983, Hurme et al. 1991a). Myonuclei are located centrally in the myotubes and cytoplasm is limited. Later the synthesis of muscle specific proteins begins and regenerating myofibers acquire a mature form with cross-striated bundles of myofilaments and peripherally located myonuclei.

Preserved, empty BMs serve as a scaffold for the reconstruction of the regenerating myofibers (Vracko and Benditt 1972). The preservation of BM, however, is not a prerequisite for the regeneration process (Caldwell et al. 1990). Empty BMs seal off fibroblasts and the majority of the newly forming collagen fibers within the BM cylinders preventing scar formation within the BM cylinders. In contrast, outside the cylinders between the injured myofiber stumps the scar formation is abundant. Blood derived fibrin and fibronectin cross-link to form a primary matrix, which acts as a scaffold and anchorage site for the invading fibroblasts and gives the initial strength to the scar to withstand the forces applied on it (Lehto et al. 1985a, Lehto and Järvinen 1985, Lehto et al. 1986, Hurme et al. 1991b). Fibroblasts begin to synthesize proteins as well as proteoglycans of the ECM. Fibronectin is expressed among the first ECM proteins followed by type III collagen. The production of type I collagen is activated later and remains elevated for several weeks. Regeneration of the damaged myofibers and formation of a connective tissue scar are two concurrent processes which are at the same time supportive but also competitive with each other (Kalimo et al. 1997). Excessive connective tissue formation may obstruct regrowth of the injured muscle.

The regenerating myofibers first fill the old BM by day 5 after the injury and then extend out of the opening of the BM into the connective tissue, attempting to pierce through the scar (Hurme et al. 1991a). Division of fibers into several small branches allows better penetration into the scar. At the same time regenerating myofibers begin to

adhere to the connective tissue on their lateral aspects and later beginning about two weeks after the injury, new mini-MTJs are formed at the ends of regenerating myofibers (Hurme and Kalimo 1992b) (see below). Gradually the intervening scar diminishes in size, and thereby stumps are brought closer to each other. Finally myofibers become interlaced. The reunion of injured myofibers after muscle shearing injury has not been observed.

3.3. Adhesion in regeneration

After muscle injury the continuity of the tendon-muscle-tendon unit is interrupted. Adhesion of myofibers to the ECM during the repair process is necessary to restore the ability of the muscle to contract as one unit. Hurme and Kalimo (1992b) have shown appearance of tenascin immunoreactivity along the sides of the regenerating myofibers both in the regenerating and surviving parts during the early regeneration process and later accentuation of the immunoreactivity at the ends of regenerating myofibers at the sites of newly formed mini-MTJs. Tenascin is an ECM protein, which in mature myofibers is found only in the MTJs providing firm anchorage of myofibers to the connective tissue (Chiquet and Fambrough 1984a and b). Thus, lateral expression of tenascin supports the necessity of firmer lateral attachment during the early regeneration process when regenerating myofibers are still growing into the scar and cannot yet be firmly attached at their ends. Hurme and Kalimo (1992b) have also shown that immunoreactivity for the integrin β1-subunit first disappears after muscle injury but then reappears at the lateral sarcolemma of regenerating myofibers as a thicker line than in the control muscle. This type of firmer lateral attachment together with the scar formation may allow the use of the muscle before regeneration is completed. Increased immunoreactivity of subsarcolemmal vinculin has also been observed after muscle contusion injury (Kami et al. 1993). Dystrophin has been shown to first disappear during myofiber necrosis and later reappear during the regeneration process (Vater et al. 1992). The exact role and sequence of expression of both dystrophin and integrin and associated adhesion molecules during muscle regeneration process after muscle shearing injury are still poorly known. Vater et al. (1995) have shown that after in situ necrosis type of muscle injury, α-SG and α-DG remain present in contrast to β-DG and dystrophin, which first disappear and later reappear during the regeneration process.

3.4. Mechanical stress and regeneration

Different types of mechanical stress have an effect on the regeneration process. Early mobilization is known to induce more rapid and intensive capillary ingrowth to the injured area, better muscle fiber regeneration and orientation of regenerating myofibers (Järvinen 1975, Järvinen 1976a, Järvinen and Lehto 1993). Mobilization also restores tensile strength sooner to the normal level (Järvinen 1976b). However, mobilization started immediately after the injury may cause reruptures at the injury site. This can be avoided by a short immobilization period, which allows the newly formed granulation tissue to reach sufficient tensile strength to withstand the forces created by muscle contraction (Järvinen 1975, Järvinen 1976b, Lehto et al. 1985a, Lehto et al. 1985b). In contrast, long immobilization may be harmful, causing significant atrophy and thus reduced tensile strength (Järvinen 1975, Järvinen 1976b, Lehto et al. 1985a). Denervation of the injured muscle eliminates neural trophic effects and contractile activity of the muscle. Innervation does not seem to be necessary for the early stages of regeneration but is an obligatory requirement for the growth and restoration of normal morphology (Mussini et al. 1987, Sesodia and Cullen 1991). Altered mechanical status obviously has an important role in the initiation and regulation of the muscle regeneration process. Mechanical stress is likely to regulate the adhesion process, which is essential in the restoration of tensile strength. The role of various adhesion molecules during different mechanical conditions in muscle repair is not known.

In biomechanical tests after contusion injury, muscles have been shown to rupture on pulling at the injury site during early muscle repair (Järvinen 1976b, Crisco et al. 1994). Studies on contusion and strain injuries have demonstrated that later failure occurs near the MTJs (Almekinders and Gilbert 1986, Taylor et al. 1993, Crisco et al. 1994). Thus, the area near the MTJs seems to be mechanically the weakest point of the musculotendinous system (Garrett et al. 1987, Garrett et al. 1988, Tidball et al. 1993, Law et al. 1995). Little is known about the correlation between these biomechanical and basic morphological changes or about adhesion during the regeneration process.

AIMS OF THE STUDY

1. To develop a reliable model of the shearing type of muscle injury.

2. To examine structural changes during the muscle regeneration process after shearing injury and analyse the significance of these changes in the restoration of tensile strength.

3. To study the re-establishment of the adhesion between the regenerating myofibers and ECM during muscle repair and correlate alterations in the expression of different adhesion molecules and their developmentally regulated alternatively spliced isoforms with the biomechanical and histopathological changes.

4. To analyse the effect of different degrees of mechanical stress on the adhesion process during muscle regeneration.

MATERIALS AND METHODS

1. Animals (I-IV)

In this study 364 male Sprague-Dawley rats were used (Table 1.). The animals were housed in cages and fed with laboratory pellets and water ad libitum. The research scheme was accepted by the ethical committee for animal experiments of the University of Tampere.

Table 1. Experimental schedule.

Study N	Subgroup	Methods	Age (wk)	Weight (g) (ave±SD)	Days post-injury
I 160	Group 1 (n=28)	Histology Immunohistochemistry	12	394 ± 27	5,7,10,14,21, 28,56
	Group 2 (n=72)	Biomechanics	12	391 ± 26	5,7,10,14,21, 28,56
	Group 3 (n=60)	Reproducibility (biomechanics) -32 traumatized rats -28 untraumatized rats	8-16	366 ± 70	14,21
II 72		Histology Immunohistochemistry Northern blotting	12	413 ± 26	2,3,4,5,7,10, 14,21,28,56
III 160	Groups: FM MO IM DE IM+DE	Histology Immunohistochemistry In situ hybridization	12	423 ± 47	3,5,7,10,14, 21,28,56
IV 50		Histology RT-PCR	12		2,3,4,5,7,10, 14,21,28,56

2. Muscle injury model (I-IV)

The animals were anesthetized either with an intraperitoneal injection of pentobarbital (Mebunat, Orion-Farmos, Turku, Finland) (I,II,IV) or with a subcutaneous injection of a combination of midazolam (Dormicum, F.Hoffmann-La Roche AG, Switzerland) and fentanyl-fluanisone (Hypnorm, Janssen-Cilag Ltd, UK) (III). Buprenorphinum (Temgesic, Reckitt&Colman, UK), given as subcutaneus injection was used as a painkiller in Study III. Under anesthesia, the lateral aspect of either right hindlimb (unilateral trauma) or of both hindlimbs (bilateral trauma) was shaved and a longitudinal incision was made through the skin and fascia along the calf. Thereafter, the soleus muscle was gently exposed to avoid detaching it from the surrounding fascia. The soleus muscle was completely transected with microscissors (S&T Marketing AG, Neuhausen, Switzerland) slightly distal to the middle part of the muscle in a transverse plane. The stumps were covered and kept aligned by suturing (Vicryl; Johnson and Johnson, Ethicon, Norderstedt, Germany) the overlying fascia. Finally, the skin was closed with stitches. The contralateral limbs of the unilaterally traumatized animals served as controls. The animals in Studies I, II and IV were allowed to move freely in the cages after the operation.

3. Post-traumatization treatment (III)

After muscle injury the animals in Study III underwent a post-traumatization treatment. The animals were divided into the following groups:

Group FM (Free Mobilization): The animals in this group were allowed to move freely in the cages after operation without any post-traumatization treatment.

Group MO (Mobilization): After muscle injury the animals were exercised each day on a motor-driven treadmill with an inclination of 10° at a constant speed (15cm/s). The training time was 10 minutes on the first day, 15 minutes on the second and third day and 20 minutes thereafter.

Group IM (Immobilization): After traumatization a padded metallic splint (Alumafoam, coNco medical company, Bridgeport, CT, USA) reaching from the toes to the knee joint

was immediately applied around the injured hindlimb with an elastic adhesive bandage reaching above the knee (Tensoplast, Smith & Nephew Medical Limited, Hull, UK). The ankle was fixed at about 10° plantar flexion. The splints were examined daily and replaced or repaired whenever necessary.

Group DE (Denervation): After muscle injury the sciatic nerve of the traumatized hindlimb was cut at midthigh level and a 10 mm segment of the nerve was removed in order to prevent reinnervation. The lesion site of the nerve was explored to check the permanence of the neurectomy when the animals were sacrificed.

Group IM+DE (Immobilization and Denervation): After transection of the soleus muscle the ipsilateral sciatic nerve was cut at the mid-thigh level and hindlimb immobilized as described above.

4. Biomechanical testing (I)

Traumatized and uninjured soleus muscles (groups 2 and 3 in Study I) were biomechanically tested using a materials testing machine (LR 5K; J. J. Lloyd Instruments, Southampton, UK). Under anesthesia the calves were prepared free from the overlying skin and the gastrocnemius muscle was removed to expose the soleus muscle. An initial fiber length was measured with a digital micrometer (Mitutoyo, Andover, UK) with the knee and ankle in 90° flexion. Thereafter the distal attachment of the muscle was released while the proximal attachment was left intact.

The animal was positioned on the platform with the tested hindlimb extended and the knee joint in 90° of flexion. The hindlimb was tightly secured to the metallic supports and the achilles tendon was then attached with a clamp to the traveling crosshead of the testing machine. The longitudinal axis of the soleus muscle was adjusted parallel to the axis of the testing machine, which is the direction of traction. The soleus muscles were passively pulled to failure at a rate of 2 mm/sec. Warm normal saline was used to keep the soleus muscles moist and at physiological temperature. During testing the load-displacement curve was recorded and the site of failure was observed. Immediately after tensile testing the soleus muscles were dissected free from the tendons, washed in saline and weighed. The animals were sacrificed after the procedure.

The following variables were determined (failure load and elongation) from the load-displacement curve or calculated (stress, specific energy and strain) using the software provided by the manufacturer:

1) failure load (N): maximum load recorded
2) elongation (mm): displacement from the initial length to the length at failure
3) stress (N/m^2): failure load (N) / physiological cross-sectional area (m^2). Physiological cross-sectional area = [muscle mass (g) x cos θ (m^2)] / [initial fiber length (cm) x muscle density (g/cm^3)]. θ = fiber pinnation angle. (Law et al. 1995, Segal et al. 1986)
4) specific energy (mJ/g): energy (mJ) (i.e. the area under the load-displacement curve to the point of failure) / muscle weight (g)
5) strain: elongation (mm) at the point of failure / initial fiber length (mm)

The fiber pinnation angle, i.e. the orientation of the muscle fibers relative to the axis of force generation, is 0-3° in rat soleus muscle (Segal et al. 1986) and therefore the cosine term was omitted in the calculations of stress ($\cos \theta \sim 1$). The density of an intact rat soleus muscle is 1.062 g/cm^3, whereas its density after 56 days of muscle grafting has been observed to decrease to 1.055 g/cm^3 (-0.7%) due to increased concentration of connective tissue (Segal et al. 1986). As regards the muscle density change after the laceration injury in our study, we anticipated that it was of the same magnitude as that observed in the study by Segal et al. (1986). Therefore, the effect of this apparently small decrease in muscle density on calculations was considered negligible, and the constant muscle density of 1.062 g/cm^3 was used in our calculations of all stress data.

5. Samples (I-IV)

Animals were sacrificed either with carbon dioxide overdose or cervical dislocation under anesthesia. The soleus muscles for histology, immunohistochemistry and in situ hybridization were collected and frozen in isopentane cooled with liquid nitrogen. After biomechanical tests samples from the proximal, distal and injured parts of the ruptured

soleus muscles were collected and frozen in liquid nitrogen for Northern blot analysis and reverse transcriptase-polymerase reaction.

6. Histology and Immunohistochemistry (I-IV)

The frozen samples were longitudinally cut into 5 μm sections and stained with hematoxylin & eosin (H&E) and Herovici for structural analysis (I-IV). Immunohistochemistry was performed with mouse monoclonal antibodies to the following molecules: α7 integrin (kindly donated by Dr. Stephen Kaufman, University of Illinois, Urbana, IL, USA) (I-III) and vinculin (Serotec Ltd, Oxford, U.K.) (II), and dystrophin 2 (II), β-DG (II), α-SG (II) and merosin (II, III) (all from Novocastra Laboratories, Newcastle, UK). The bound antibodies were visualized using avidin-biotin peroxidase kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen and hematoxylin as the counterstain.

6.1. Quantification of immunohistochemistry (II,III)

Immunohistochemical samples were quantified by measuring the maximal intensity (relative optical density, ROD) of the immunoreactivity associated with the sarcolemma or BM with Microcomputer Imaging Device version M4 (Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada). Measurements were performed on the digital pictures captured by scanning the intensity of immunopositivity in a band of selected width, which is perpendicular to the plasma membrane and BM. The staining of sarcoplasm was also measured. Measurements in each sample were made in ten different areas.

Immunoreactivity was measured both in the regenerating part of the muscle [i.e. in the regeneration and central zones, (RZ and CZ)] and in the intact muscle [i.e. both in the survival zone (SZ) and in the contralateral control muscle] (Fig. 3A-C). For reliable quantification sections from the traumatized and control muscle were immunostained on the same slide. The intensity of immunoreactivity of each molecule in the RZ and CZ of the regenerating myofibers was expressed as a percentage of the intensity in intact muscle (i.e. in either SZ or control muscle) and the immunoreactivity in the SZ of the regenerating muscle as a percentage of the control muscle.

The average value of the non-specific staining of sarcoplasm in the intact area was used as the backround error value (BEV). The BEV in the same section was subtracted from the average intensity of immunostaining of the plasma membrane or BM in both the regenerating and intact area (see formula). The formula of the calculation used was:

Intensity of true immunoreactivity (%) =

$$100 \times (ROD_{regeneration} - BEV_{intact})/(ROD_{intact} - BEV_{intact}).$$

ROD_{intact} represents the intensity of immunoreactivity either in the SZ of the same tissue section (for dystrophin and associated molecules) or in a control section from the contralateral muscle immunostained on the same slide (for integrin and associated molecules). Contralateral intact muscles had to be used as reference values for integrin and associated molecules because trauma induced upregulation occurred in the SZ.

7. Northern blot analysis (II)

Total cellular RNA was isolated using the guanidium thiocyanate/CsCl method (Chirgwin et al. 1979). Twenty μg of total cellular RNA was separated in 1% agarose gels containing formaldehyde, transferred to nylon membranes (ZETA-probe, Biorad), and hybridized with ^{32}P-labeled (Amersham) cDNAs for rat α1 (I) collagen (Genovese et al. 1984), rat β1 integrin, rabbit dystroglycan (Ibraghimov-Beskrovnaya et al. 1992) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a "housekeeping" enzyme used as a control) (Fort et al. 1985) probes. Rat β1 integrin cDNA corresponding to nucleotides 45 - 994 (unpublished sequence in Gene Bank, accession number U12309) was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA isolated from rat granulation tissue cells. Dystroglycan cDNA was kindly donated by Dr. Kevin Campbell (Iowa City, Iowa, USA). Microcomputer Imaging Device version M4 was used to quantify autoradiograms and results were corrected for GAPDH mRNA levels.

8. Messenger RNA in situ hybridization (III)

In situ hybridization was carried out as previously described (Kononen and Pelto-Huikko). Two oligonucleotide probes directed against the rat α7 integrin A/B chains (nucleotides 3214-3247 and 3570-3603; GenBank accession number X65036) (Song et al. 1992) were used. When compared with the known sequences in the GenBank database, sequences exhibited less than 80% homology with any other known gene. Both probes produced similar results when used separately and were usually combined to intensify the hybridization signal. Several probes to non-related mRNAs with known expression patterns, with similar length and GC-content, were used as controls to verify the specificity of the hybridizations. The probes were labeled to specific activity of 1 x 10^9 cpm/µg with ^{33}P-dATP (DuPont-NEN, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Int., Buckinghamshire, UK). The sections were briefly air dried and hybridized at 42°C for 18 h with 5 ngm/ml of the probe in a mixture containing 4 x SSC (1 x SSC=0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 1x Denhardt´s solution (0.02% polyvinyl-pyrroline, 0.02% bovine serum albumin and 0.02% Ficoll), 1% sarkosyl, 0.02 M phosphate buffer (pH 7.0) and 10% dextran sulphate. After hybridization, the sections were rinsed 4 times at 55°C in 1 x SSC for 15 min each and then left to cool for 1 h at room temperature. The sections were dipped in distilled water, dehydrated with 60% and 90% ethanol and air dried. Thereafter the sections were covered with Kodak MR5 autoradiography film (Kodak, Rochester, NY, USA) and exposed at -20°C for 30 to 60 days. The autoradiography films were developed using LX24 developer and AL4 fixative (Kodak).

8.1. Quantification of in situ hybridization

Quantification of in situ hybridization results was performed with the image analysis system consisting of IBM-PC, SensiCam digital camera (PCO Computer Optics GmbH, Kelheim, Germany), a Nikon 55 mm lens and Northern Light precision illuminator (Imaging Research, St. Catharines, Ontario, Canada). Image-Pro Plus program (Media Cybernetics, Silver Spring, MD, USA) was used for the measurements. The gray levels corresponding to ^{14}C-plastic standards (Amersham, Buckingshamshire, UK) lying within the exposure range of the film were determined and used in a third degree polynomial (Lagrange) approximation to construct the gray level to activity transfer function. The borders of the measuring fields were interactively defined, and the

average activity in the tissue fields was calculated. mRNA levels were measured both in the intact and regenerating parts of the muscle and relative changes in mRNA levels were expressed as percentage of control. Sections from four animals in each group were measured, and the mean values were used in the statistical analysis.

9. Reverse transcriptase-polymerase chain reaction (RT-PCR) (IV)

Total RNA from muscle samples was isolated using the guanidium thiocyanate/CsCl method (Chirgwin et al. 1979). Reverse transcription and subsequent PCR were carried out with 1.0 μg of total RNA using Gene Amp PCR kit (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ, USA). Synthesized cDNAs were amplified by PCR in Perkin Elmer DNA Thermal Cycler. Primers used in the amplification of β1A and β1D variants of cytoplasmic domain were NZ1 (5′-TTGTGGAGACTCCAGACTGTCCTACT-3′) and PE6 (5′-TCATTTTCCCTCATACTTCGGATT-3′) (designed from Argraves et al. 1987 and Holers et al. 1989); primers for amplification of α7A and α7B variants of cytoplasmic domain were 3154 (5′-GTTGTGGAAGGAGTCCC-3′) and 3155 (5′-GTCTTCCCGAGGGATCTT-3′) (designed from Collo et al. 1993); primers for amplification of α7X1 variant of extracellular domain were 5′-CTATCCTTGCGCAGAATGAC-3′ and 5′-GCCAGGGTGGAGCTCTG-3′and primers for α7X2 variant were 5′-CTATCCTTGCGCAGAATGAC-3′ and 5′-GTGACCAACATTGATAGCTC-3′(designed from Ziober et al. 1993) (CyberGene AB, Huddinge, Sweden). NZ1 and PE6 oligonucleotide primers flank the region where β1A and β1D splicing variants differ from each other, i.e., β1D contains a specific 81 bp insertion compared to β1A. α7A contains alternatively spliced 113 bp segment, which is not present in α7B variant. PCR products were analysed on 1.5 (X1/X2) or 2% agarose gel stained with ethidium bromide, using a 100-bp ladder as a standard. The intensity of bands was quantified using Microcomputer Imaging Device version M4 (Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) and the relative proportion of the intensity of each splicing variant was expressed as a percentage of the total intensity of both bands at each time point.

10. Statistical analysis (I-III)

Values for failure load, stress, strain, specific energy and muscle weight for the control and injured muscles (group 2 in Study I) were analyzed using a paired *t*-test. Due to seven comparisons, a p-value ≤ 0.007 was considered significant. The analysis of variance (ANOVA), followed by Bonferroni correction for multiple comparisons, was performed to compare biomechanical results (I) and the intensities of immunoreactivities of each molecule (II) between different healing time points. Analysis of variance was also used to compare the intensities of immunoreactivities between different molecules (II) and to compare the mRNA levels and intensities of immunoreactivities between different post-traumatization treatment groups (III) at each healing time point. A p-value <0.05 was considered significant.

The reproducibilities of the tensile testing (I) and the quantification of immunohistochemistry by image analysis (II) were determined by comparing biomechanical data from the ipsilateral and contralateral soleus muscles (group 3 in study I) and the intensities of immunoreactivity from repeated measurements on different immunohistochemical sections (dystrophin staining) (II). Reproducibility was defined as the 95% limit of agreement for the differences observed in interlimb measurements (I) and the differences observed in repeated measurements on different sections (II) (average bias ± twice the SD of the differences). The 95% limit of agreement was used to make certain that the observed differences were significantly larger than those occuring by chance.

RESULTS

1. Morphological changes during muscle regeneration (I-IV)

The histopathological pattern after muscle shearing injury followed the same scheme as previously described (Hurme et al. 1991a). In short, after muscle injury the ruptured myofibers retracted and the gap between the stumps formed the central zone (CZ) (Fig. 3A). CZ was first filled by a hematoma and was later replaced by connective tissue. Ruptured myofibers became necrotized from the site of transection over a distance of 1-2 mm inside their preserved BM cylinders. This part is called the regeneration zone (RZ). The formation of demarcation membrane limited the extension of necrosis and delineated the RZ from the survival zone (SZ), where myofibers survived with certain reactive changes.

Satellite cells had proliferated and become myoblasts by day 2 and fusion into multinucleated myotubes had started by day 3. Regenerating myofibers filled the old BM cylinders by day 5 and thereafter started to pierce through the scar between the muscle stumps. Gradually the regenerating myofibers acquired their mature form with peripherally located myonuclei and with bundles of myofilaments in register giving the myofiber its cross-striated appearance. There was a gradient in the proximal and distal parts of the regenerating myofibers as maturation in the proximal parts was always ahead of that in the distal parts. The connective tissue scar contracted and the stumps of regenerating myofibers were pulled closer to each other. Formation of new MTJs at the ends of regenerating myofibers was observed from day 14 onwards.

2. Biomechanics during muscle regeneration (II)

Muscle weight in traumatized muscles decreased significantly from days 21 to 56 after muscle injury to an approximate level of 60 % of the control muscles. The failure load increased from day 5 to day 14, but then remained at about the 50 % level of the control values until the end of the experiment. The breaking stress of the injured muscles gradually increased from day 5 to day 21 to approximately 85 % level of the control value and finally almost reached the control level by day 56. The strain increased from day 5 to day 14 to about 60 % of the level of the control value and then slightly decreased and remained at a level 50 % of the control muscles. The specific energy of

the injured soleus muscles increased from day 5 to day 10 to a level of about 35 % of the control value and thereafter remained about at the same level until day 56.

During the mechanical testing, the control muscles ruptured near the proximal or distal MTJs. The traumatized regenerating soleus muscles ruptured in the scar tissue at the site of injury on days 5 and 7. On day 10 the failure occurred either at the site of injury or close to it within intact myofibers. After day 10 the failure occurred within intact myofibers either close to the site of injury or close to the intact MTJs.

2.1. Reproducibility of the biomechanical testing

The 95 % limits of agreement for the biomechanical variables of uninjured animals were of about the same magnitude or even wider than the SDs of the variables (I, Table 1). This means that the inherent interlimb variability compares well with the interanimal variability observed for the same property. Traumatization seemed to increase (doubled) the variability regarding the reproducibility of failure load and stress.

3. Adhesion of regenerating myofibers (I-IV)

3.1. β1 integrin, α7 integrin and vinculin

Northern blot analysis of injured soleus muscles showed a 4-fold upregulation of integrin β1 mRNA levels by day 5 after muscle shearing injury (II). Integrin β1 mRNA levels returned to control levels by day 10. The in situ hybridization analysis in Study III (group FM) demonstrated increased expression of α7A/B mRNA both in the intact and regenerating parts of the injured myofibers. Maximum intensity of about 170 % of the control value was reached in the intact part on days 5-7 and a maximum value of about 740 % in the regenerating part on day 3. The intensity of α7A/B mRNA level rapidly decreased both in the intact and regenerating parts of the myofibers and approached the control intensity by days 28-56.

Immunoreactivity for α7 integrin (II, III: group FM) and vinculin (II) in the control muscles was observed as a strong immunostaining in the MTJs whereas the plasma membranes stained weakly (Fig. 3A).

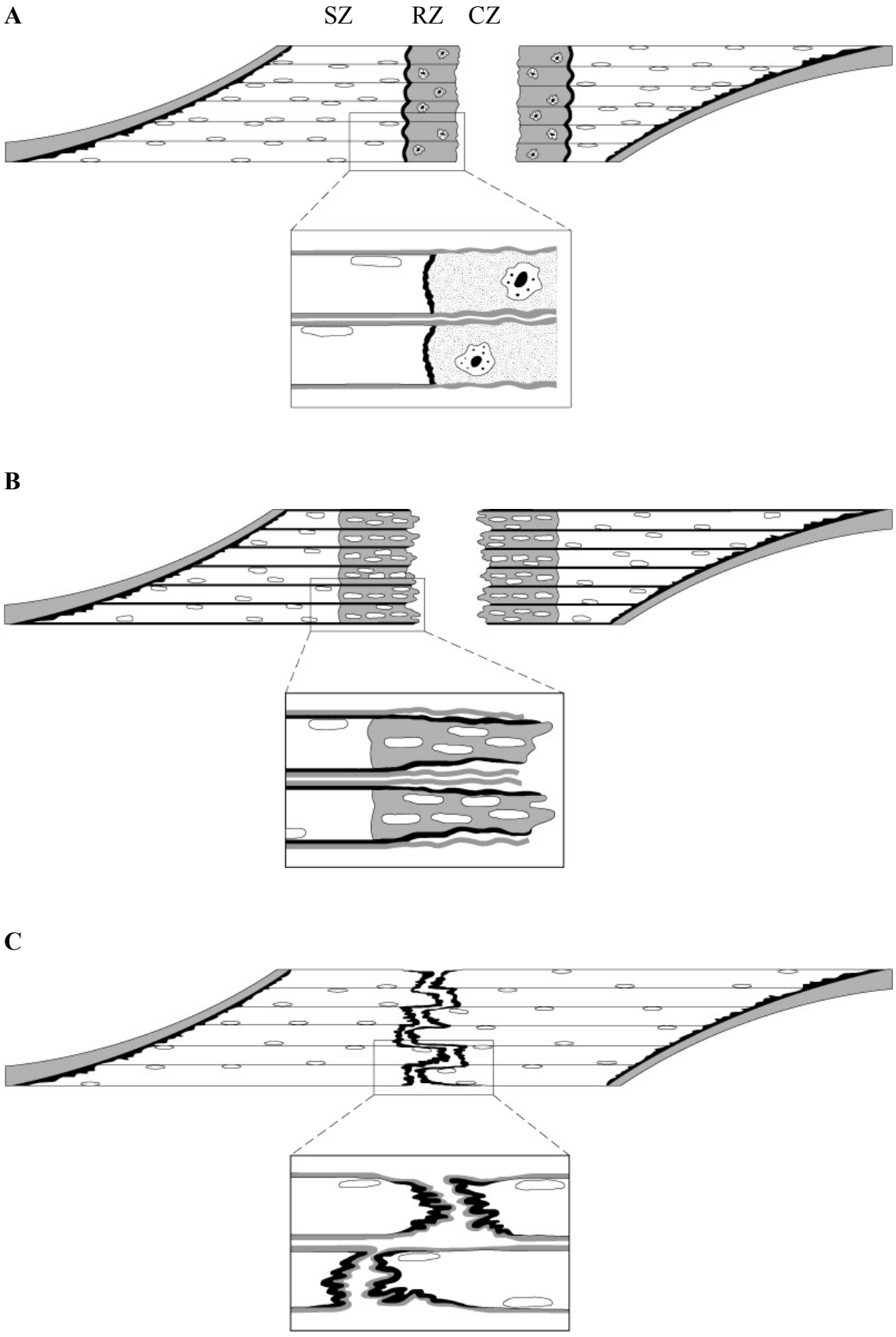
A
SZ
RZ
CZ
B
C

Figure 3. A-C. Schematic illustrations of α7 integrin immunoreactivity in the lesioned soleus muscles during the regeneration process. (A) In the beginning of the repair process the α7 integrin immunoreactivity is corresponded to that of control muscle, i.e. immunoreactivity is enriched in the MTJs with only minor amounts being present on the lateral aspects of the myofiber plasma membrane. (B) During days 5-7 after injury increased immunoreactivity was observed along the lateral aspect of the plasma membrane both in the intact (SZ) and regenerating parts (RZ, CZ) of the injured myofibers. (C) Later in the repair process the immunoreactivity in the lateral sarcolemma returned to the control level with simultaneous redistribution of α7 integrin to the ends of the regenerating myofibers to the newly formed MTJs.

During the early regeneration process the intensity of α7 integrin (II, III: group FM) and vinculin (II) immunoreactivities increased along the lateral aspect of the plasma membrane both in the intact (SZ) and regenerating parts (RZ, CZ) of the injured myofibers (Fig. 3B). Increased intensity of α7 integrin immunoreactivity was similar along the whole length of the myofibers in SZ and reached a maximum value of about 170-180 % on day 7. The maximum intensity of about 175 % in the regenerating part of the myofibers was reached by days 5-7. The increase in the intensity of vinculin immunoreactivity in the SZ was most marked next to the injury site reaching a maximum of about 185 % on day 7, whereas farther away from the lesion, the maximum increase was only about 120 % on day 5. In the regenerating part of the myofibers the vinculin immunoreactivity reached a maximum of about 240 % by day 5. The α7 integrin and vinculin immunoreactivity in SZ returned to the control level by day 10. In the regenerating part of the myofibers the immunoreactivity for α7 integrin returned to the control level by day 14 and the immunoreactivity for vinculin by day 28. The return of lateral α7 integrin immunoreactivity to control levels was associated with simultaneous redistribution of α7 integrin to the ends of the regenerating myofibers, where the formation of new MTJs began around day 14 (I-III) (Fig. 3C). Vinculin immunoreactivity also became accentuated at the ends of regenerating myofibers.

3.2. Splicing variants of β1 integrin and α7 integrin

The relative level of β1D transcript analysed by RT-PCR (IV) was 0 % to 11 % during days 2-21 after muscle injury and correspondingly the level of β1A transcript was 100 to 89 %. The relative amount of β1D isoform rapidly increased after day 21 reaching the 51 % level on day 56 while the relative amount of β1A decreased. The relative level of β1D isoform on day 56 was slightly lower than in the control tissue (65 %). The relative

level of α7A transcript increased from day 2 (15 %) to day 4 to a level of 96 % while the relative amount of α7B transcript decreased. After day 4 the level of α7A transcript gradually decreased and the relative level of α7B transcript increased until day 56 to levels of 8 % and 92 %, respectively. The ratio of α7A and α7B transcripts in the control tissue was approximately the same as on day 56. The relative level of α7X1 transcript increased from day 2 (51 %) to day 4 to a level of 72 %, whereafter it gradually decreased to the level of 38 % on day 10. From day 10 to day 56 the value of α7X1 transcript was only slightly changed. On day 56 the levels of α7X1 and α7X2 isoforms were 39 % and 61 % respectively. In the control muscle the corresponding values for α7X1 and α7X2 isoforms were 40 % and 60 %.

3.3. Dystrophin, β-dystroglycan and α-sarcoglycan

Immunoreactivities for dystrophin, β-DG and α-SG in the control muscles and in the SZ of the regenerating myofibers were identified as a distinct continuous line delineating the plasma membrane (II). In the regenerating part of the myofibers the onset of dystrophin expression on days 3 and 4 was slightly delayed compared to that of β-DG and α-SG but after day 5 it was ahead of them with α-SG being the slowest to recover (II). The dystrophin immunoreactivity in the regenerating part reached a 50 % level compared to intensity in SZ by approximately 8 days while β-DG and α-SG did not reach a 50 % level until days 17 and 22, respectively. The intensities of dystrophin, β-DG and α-SG immunoreactivities all almost reached the control level by the end of the observation period. When the reproducibility of the quantification of immunochemistry was determined, the 95 % limits of agreement for the repeated measurement of dystrophin sections by image analyser were 26 % and 19 % (II).

3.4. Merosin

Immunoreactivity for merosin in the BM in both the control muscles and in the SZ of injured myofibers was identified as a distinct continuous line (II, III). During the early regeneration process merosin antibody stained the preserved, old corrugated BM. On days 4-5 after muscle injury another line immunopositive for merosin was detected inside the old BMs as a sign of formation of new BMs (II, III). The expression of merosin during the first 21 days was parallel to that of β-DG reaching the 50 % intensity

of the corresponding value in SZ on approximately day 17. Thereafter the merosin immunoreactivity slowly increased approaching the control level by day 56.

4. Mechanical stress and adhesion process (III)

α7A/B mRNA expressions in the intact and regenerating parts of the injured myofibers in groups MO, DE, IM and DE+IM were similarly upregulated as in the FM group after muscle shearing injury. The maximum value in these groups in the intact part (range 167-244 % of the control value) was reached on days 3-7 and in the regenerating part on day 3 (range 558-864 %). α7A/B mRNA expression in the intact and regenerating parts of the myofibers in all groups gradually decreased and approached the control intensity on days 21-56.

The α7 integrin immunoreactivity in group MO corresponded to the immunoreactivity in group FM, i.e. increased intensity was observed both in the intact and regenerating parts of the injured myofibers. A peak intensity of about 150-160 % was reached both in the intact and regenerating parts on day 3-5. However, enhancement of α7 integrin immunoreactivity in the intact and regenerating parts of the injured myofibers did not occur in groups IM, DE and DE+IM. Altered mechanical stress did not cause any significant changes in the intensity of merosin immunoreactivity.

DISCUSSION

1. Muscle injury model

The contribution of various tissue elements, such as different connective tissue components and adhesion molecules, to the restoration of tensile properties of injured skeletal muscle are difficult to analyse in partial laceration (Hall-Craggs 1974, Garrett et al. 1984, McGeachie and Grounds 1987, McGeachie and Grounds 1995), strain (Taylor et al. 1993) and contusion (Järvinen 1975) injury models where part of the muscle remains uninjured. Therefore, a new model of muscle injury with complete transection of the soleus muscle was developed. The soleus muscle, as a unipennate muscle with tendons only at its ends, is easily accessible and can be completely cut. Its anatomy guarantees that transection will cut only myofibers. In addition, sharp transection ensures a better orientation of regenerating myofibers, which makes the analysis of the repair process easier. It is realized that in clinical situations muscle injuries are hardly ever as clean-cut as in our injury model but the basic phenomena of the muscle regeneration are likely to be similar. Our injury model makes it possible to relate the biological changes to the mechanical changes during muscle repair after the shearing type of muscle injury. The reproducibility of both the traumatization method and the mechanical testing were found sufficient considering the large interlimb and intergroup differences observed in the experimental groups.

2. Morphological changes and biomechanics

The morphological results of the present study demonstrated the excellent capacity of muscle to recover after muscle shearing injury. Transected soleus muscles were shown to restore their structural integrity and the functional continuity of the muscle across the gap between the stumps, i.e., regenerating myofibers were able to pierce through the connective tissue scar finally forming a well organized structure of interlacing myofibers with little connective tissue between the fibers. The ends of the regenerating myofibers were shown to bind to the scar tissue by forming new MTJs from day 14 onward, as shown immunohistochemically (see below) in this study and as previously demonstrated ultrastructurally (Hurme et al. 1991a, Hurme and Kalimo 1992b). Reunion of the stumps of the regenerating myofibers was not detected during the observation period of this study. A reunion does not seem to occur even after months,

since strongly α7 immunopositive ends (MTJs) and intervening scar were still detectable (Vaittinen et al. unpublished observation) and it may be that the myofibers remain permanently divided in two parts.

Connective tissue scar between the muscle stumps was found to be the weakest point in the traumatized soleus muscles until day 10 after injury, because during that time mechanical failure occurred in the scar, in agreement with the earlier results also demonstrated in contusion injury models (Järvinen 1976b, Crisco et al. 1994). Thus, biomechanical results with rapidly increased failure load, stress, strain and specific energy values during that period characterized the restoration of the tensile strength of the scar and re-establishment of the lateral adhesion of regenerating myofibers to the scar (see below).

After day 10 the failure in the traumatized regenerating muscles occurred within the myofibers either near the injury site or the intact MTJs, which was also the rupture site in the control muscles. Changing of the failure site coincided with the formation of new MTJs at the ends of regenerating myofibers as well as with the transformation of the loose connective tissue into a stiffer and stronger scar. Strain and contusion injury studies have also demonstrated mechanical failure to occur near the MTJs during the late phase of muscle repair (Almekinders and Gilbert 1986, Taylor et al. 1993, Crisco et al. 1994). Our findings thus support the current view of considering the myofibers near the MTJ to be mechanically the weakest point of the musculotendinous system (Garrett et al. 1987, Garrett et al. 1988, Tidball et al. 1993, Law et al. 1995). From day 14 onwards the traumatized muscles atrophied significantly. During that time, the failure load, strain and specific energy values were not increased in contrast to the increase in the stress value. This indicates that the regenerated muscle, with respect to its reduced size, had recovered in tensile strength almost to the control level and active mobilization therapy is needed to restore the normal size and thus normal strength of the muscle.

3. Adhesion of regenerating myofibers

In a shearing type of muscle injury the connective tissue sheaths are also ruptured. Thus, the attachment of regenerating myofibers to the ECM, in other words the integrin and dystrophin mediated connection between the contractile proteins and ECM, has to

be re-established to restore the functional continuity and tensile strength of the traumatized regenerating muscle. Quantitative immunohistochemistry offered the only method allowing analysis of these molecules at their functional site in all three zones (CZ, RZ and SZ) of regeneration. An internal standard stained on the same slide was always used in the calculation of relative intensities (%) to make the quantification of immunohistochemistry most reliable. The reproducibility of the quantification was found good considering the marked intensity changes reported in the experimental groups. In addition to immunohistochemistry, Northern blot analysis, in situ hybridization and RT-PCR were used to investigate the adhesion process.

The expression of α7 integrin in the regenerating parts of the injured myofibers was upregulated both at the gene and protein product level during the early repair process after muscle shearing injury. Upregulation of β1 integrin was also demonstrated at the mRNA level in our study and immunohistochemically in the earlier muscle contusion injury study by Hurme and Kalimo (1992b). The α7 integrin mRNA levels in the regenerating part of the myofibers were markedly higher than the protein levels. Active transcription in this area is logical, because the density of nuclei in regenerating myotubes is exceptionally high and possibly excessive in proportion to the extent of newly synthesized sarcolemma where integrin could be deposited. It is possible that not all transcripts in the regenerating part are not translated or that the newly translated integrins are not transported to the cell surface. The later possibility is supported by the presence of cytoplasmic α7 integrin immunoreactivity. In addition, the halflife of α7 integrin may be reduced in the regenerating part. Upregulation of α7 integrin mRNA levels were also observed to a lesser extent in the surviving part of the myofibers during the early repair process, but in contrast to the regenerating part the increase in transcription was of similar magnitude as in translation.

Increased expression of β1 integrin and α7 integrin mRNAs and subsequent accentuation in the intensity of α7 integrin and vinculin immunoreactivities on the lateral sarcolemma of intact and regenerating parts of the myofibers during the early repair process indicate reinforced lateral adhesion of the regenerating myofibers to the ECM. During this time period the regenerating myofibers are still growing into the scar tissue and their ends cannot yet firmly bind to the ECM. This reinforced lateral adhesion

most likely contributes to the improved tensile strength by reducing the movements of the stumps and pull on the still fragile scar, and thus apparently reduces the risk of rerupture. Functionally this is also important, because lateral adhesion allows the use of the injured muscle before the healing is complete. Upregulation of integrin expression may also have a signaling role related to the induction of adaptive metabolic processes needed to respond to altered biomechanical state after the muscle transection (Chiquet 1999, Giancotti and Ruoslahti 1999).

Later during the repair process the β1 integrin and α7 integrin mRNAs levels and α7 integrin and vinculin immunoreactivities on the lateral aspects of regenerating myofibers are normalized with simultaneous accentuation of α7 integrin and vinculin immunoreactivities at the ends of regenerating myofibers. These events coincide with the formation of new MTJs at the ends of regenerating myofibers. It seems logical that during the re-establishment of firm terminal adhesion, reinforced lateral adhesion is no longer needed, and α7 integrin and vinculin molecules most likely move along the plasma membrane to the new MTJs at the ends of regenerating myofibers. Such a lateral movement possibly guided by ligand binding has earlier been described for β1 integrins (Felsenfeld et al. 1996). In our muscle injury model the increase in tensile strength with simultaneous decrease in the elasticity of the scar during the repair process increases the mechanical stress on the regenerating fiber, which may be one of the signals leading to the transfer of integrin molecules to the new MTJs. Tenascin-C, which is a major ECM protein in tendon, is one of the ligands of α8 integrin (Varnum-Finney et al. 1995). Thus, it seems likely that α8 integrin also has a role in myofiber-ECM adhesion in skeletal muscle. α8 integrin has been detected in smooth muscle (Schnapp et al. 1995), but its expression and localization in regenerating skeletal muscle is unknown.

The sequence of expression of β1 integrin splicing variants during the regeneration process correlate well with the changes in adhesive situations. β1A isoform, which is known to have weaker interaction with actin cytoskeleton compared to β1D isoform (Belkin et al. 1997), is expressed at a higher level during the early regeneration process. This seems logical, because during this dynamic adhesion stage the regenerating myofibers are still growing into the scar as described in detail above. Besides, the cytoskeletal myofilaments are not yet well organized into sarcomeres. Later, the

expression of β1D isoform is increased simultaneously with the formation of new MTJs at the ends of regenerating myofibers. In addition, after MTJ formation the traumatized regenerating muscles rupture close to the newly formed MTJs or to the intact MTJs. These results are consistent with the suggested important role for β1D isoform in forming extremely stable association with cytoskeletal proteins required in muscle contraction (Belkin et al. 1997). Our results thus suggest that alternative splicing of β1 integrin most likely modulates adhesive function of integrins during muscle regeneration process. α7B was the predominant isoform in the beginning of the repair process, whereafter its level was drastically decreased and the level of α7A correspondingly increased until day 4. After day 4 the expression of α7B was gradually increased again becoming the predominant isoform in highly regenerated myofibers. These results suggest that α7B isoform is responsible for the adhesion of myofibers to ECM in mature rat skeletal muscle. α7X1 and α7X2 isoforms were both expressed throughout the regeneration process. A peak in the expression of α7X1 isoform was observed on day 4, coinciding with the dynamic adhesion stage.

The expression of dystrophin and associated proteins after muscle shearing injury followed the sequence of expression also reported during the development of skeletal muscle (Tomè et al. 1994, Mora et al. 1996), i.e., the expression of subsarcolemmal proteins preceded that of transmembrane and extracellular proteins. This suggests an important role of subsarcolemmal proteins in the assembly of transmembrane and extracellular components. In contrast, after in situ necrosis type of muscle injury transmembrane β-DG was found to reappear before subsarcolemmal dystrophin (Vater et al. 1995). In the in situ necrosis type of muscle injury the BM and mysial sheaths remain intact as opposed to disrupted BM in muscle shearing injury. This difference may have an effect on the sequence of molecular expression and thus explain the discrepancy between these two muscle injury models.

Dystrophin and associated proteins appeared later during the repair process compared to α7 integrin and vinculin. Within two weeks after muscle injury the accentuation of both dystrophin and integrin associated molecules was observed in the newly formed MTJs. During that time the immunoreactivities for dystrophin and associated molecules on the

lateral sarcolemma of regenerating myofibers increased to a higher level while the immunoreactivities for α7 integrin and vinculin decreased. This suggests that these two complexes of adhesion molecules have complementary roles in myofiber-ECM adhesion. A complementary role is also supported by earlier studies demonstrating enhanced expression of α7 integrin in the muscle of *mdx* mouse and in patients with Duchenne or Becker muscular dystrophy, which all have dystrophin deficiency (Hodges et al. 1997). Enhanced expression of vinculin has also been observed in the *mdx* mouse (Law et al. 1994).

4. Mechanical stress and adhesion process

Altered mechanical stress after muscle shearing injury did not have any effect on α7 integrin expression at the mRNA level, i.e., the muscle regeneration process itself seems to stimulate the transcription of mRNA independently of muscle activity and/or innervation. However, at the protein level enhanced expression of α7 integrin in regenerating and intact parts of the myofibers was found in the active groups (FM and MO) but not in the inactive groups (IM, DE and IM+DE). These results imply that active mechanical stimulation during the regeneration process reinforces early lateral adhesion mediated by integrins. The stimulatory effect of the spontaneous movement in the FM group is intense enough for the enhanced expression. Thus, it may be that also in humans relatively low intensity exercise after muscle injury is sufficient for the enhanced integrin expression. On the other hand, the neural trophic effect and the possible isometric muscle activity in the IM group or passive moving of the muscle in the DE group are not strong enough signals to cause enhanced expression of α7 integrin. However, denervation and/or immobilization of the traumatized muscle do not influence the base level expression of α7 integrin, because all the inactive groups reached the α7 integrin level corresponding to that of the control muscle.

SUMMARY AND CONCLUSIONS

In this study, the restoration of tensile strength, structural changes and re-establishment of myofiber-ECM adhesion during the muscle regeneration process were analysed. An injury model of sharp transection of rat soleus muscle was developed to create a shearing type of muscle injury. The regeneration process was followed from 2 to 56 days whereafter the muscles were mechanically tested and muscle samples were analysed by histological and immunohistochemical staining methods, as well as by Northern blot, in situ hybridization and RT-PCR analysis. The major findings and conclusions based on them are:

1. Complete transection of the soleus muscle in the new injury model guarantees injury only in pure muscle tissue and thus makes it possible to analyse the relationship between structural and biomechanical changes without confounding effects. The repeatability of the injury model as well as the mechanical testing were analysed and found sufficient.

2. Injured myofibers after the shearing type of injury have an extensive capacity to regenerate. Regenerating myofibers are able to restore their structural integrity. They grow into the scar between the injured myofiberstumps and form a well organized structure of interlacing myofibers and connective tissue scar thus re-establishing the functional continuity of the muscle.

Biomechanical testing showed that the connective tissue scar between the muscle stumps is the rupture site and thus the weakest point in the injured soleus muscles until day 10 after injury. Thereafter the new MTJs are formed at the ends of regenerating myofibers and the myofibers near the newly formed MTJs or intact MTJs become the weakest point and thus the rupture site of the injured muscle.

3. During the early repair process regenerating myofibers reinforce their adhesion to the ECM on their lateral aspects both in the intact and regenerating parts of the myofibers, as indicated by the increased expression of $\alpha 7$ and $\beta 1$ integrin mRNAs and accentuation of $\alpha 7$ integrin and vinculin immunoreactivities on the lateral sarcolemma. Reinforced lateral adhesion is necessary to re-establish the functional continuity of the muscle,

because the ends of the regenerating myofibers are still growing into the scar and thus cannot bind firmly at their ends. From day 14 onwards the new MTJs are formed at the ends of regenerating myofibers and reinforced lateral adhesion is no longer needed. During that time the immunoreactivities for vinculin and α7 integrin at the lateral sarcolemma decrease and the intensity of dystrophin and associated proteins increase suggesting these two complexes of adhesion molecules to have a complementary role in myofiber-ECM adhesion. Both molecule complexes are clustered in the newly formed MTJs.

β1A integrin, which has been shown to have a role in dynamic adhesion situations, is the dominant splicing variant expressed during the early muscle repair process. Later, simultaneously with the formation of new MTJs at the ends of regenerating myofibers β1A integrin is replaced by β1D integrin, which is known to form stable associations. A peak in the expression of α7X1 isoform was observed during the early repair process coinciding with the dynamic adhesion stage. α7B isoform became the predominant isoform in the highly regenerated muscle while the level of α7A was low. This suggests that alternative splicing of β1 and α7 integrins may modulate adhesive function of integrins during muscle regeneration process.

4. Increased immunoreactivity for α7 integrin on the lateral sarcolemma of regenerating myofibers was found in the active groups but not in the inactive groups during the early repair process. This indicates that active mechanical stimulation reinforces lateral integrin mediated adhesion.

ACKNOWLEDGEMENTS

The present study was carried out at the Department of Surgery, Medical School, University of Tampere and at the Department of Pathology, University of Turku, Finland, during the years 1994-2000. I wish to express my gratitude to the Heads of these departments Professor Markku Järvinen, M.D., and Professor Timo Nevalainen, M.D., for providing excellent facilities to perform the studies.

My deepest gratitude is due to my two supervisors, Professor Markku Järvinen, M.D., Head of the Department of Surgery, Tampere, and Professor Hannu Kalimo, M.D., Department of Pathology, Turku, for introducing me to the fascinating world of science. Your excellent guidance and continuous encouragement have enabled me to complete this thesis. It has been a great priviledge to work under your supervision and above all to enjoy your friendship.

I am grateful to the official reviewers of this study, Docent Timo Hurme, M.D., Department of Pediatric Surgery, University of Turku, and Professor Anders Oldfors, M.D., Institute of Laboratory Medicine, Sahlgrenska University Hospital, Göteborg, for their constructive criticism and valuable suggestions regarding the manuscript.

I am also grateful to the co-authors of my original studies, Teppo L.N. Järvinen, M.D., Ph.D., Janne Kääriäinen, M.D., Docent Harri Sievänen, D.Sc,. Professor Jyrki Heino, M.D., Liisa Nissinen, M.Sc., and Tiina Liljamo, B.M. I am very grateful for the priviledge of working in the laboratory of Professor Jyrki Heino, Medicity Research Laboratory, Turku. Especially, I want to thank Teppo Järvinen for his guidance in the practical matters during the early stages of this study and for his optimism and encouragement during the difficult periods in the research.

The excellent technical assistance of Ms. Liisa Lempiäinen, Ms. Liisi Salomaa and Ms. Heidi Pakarinen, and photography of Mr. Jaakko Liippo are gratefully acknowledged. Ms. Elena Rybnikova is thanked for consultation in the quantification of in situ hybridization experiments and Mr. Rolf Sara for guidance in the quantification of immunohistochemical preparations. The Animal Laboratory, University of Tampere, is acknowledged for their help throughout my work.

I also want to thank Mika Paavola, M.D., and Petteri Kousa, M.D., with whom I shared my office, for their friendship and support.

Virginia Mattila, M.A., is thanked for careful revision of the English language of the thesis manuscript.

My sincere thanks are due to my family and friends for background support and interest in my work throughout these years.

Finally, my warmest thanks are due to my husband, Janne, for the greatest support and loving care throughout the study. He, together with our dear sons Santeri and Julius, have shared the great moments as well as stormy days during the study and encouraged me to complete this thesis.

This study was financially supported by grants from the Emil Aaltonen Foundation, the Foundation for Orthopaedical and Traumatological Research in Finland, the Medical Research Funds of Tampere and Turku University Hospitals, the Research Council for Physical Education and Sport, Ministry of Education, Finland, the Juho Vainio Foundation, The Finnish Medical Society Duodecim and the Scientific Foundation of the City of Tampere. Permissions to reproduce the original articles were kindly granted by Elsevier Science and Journal of Orthopaedic Research.

Tampere, February 2001

Minna Kääriäinen

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