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RE-EVALUATION OF EXERCISE-INDUCED MUSCLE SORENESS

An Immunohistochemical and Ultrastructural Study

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Cover picture: A longitudinal section of the soleus muscle from an exercised subject showing extra-sarcomeres revealed by staining for alpha-actinin (green) and desmin (green)

To my family

In memory of my father

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ABSTRACT

Delayed onset muscle soreness (DOMS) is a familiar experience for the elite and novice athletes. Symptoms can range from muscle tenderness to severe deliberating pain. It is generally believed that eccentric contractions produce higher tension on muscle fibres and connective tissues than concentric and isometric contractions. This higher mechanical stress induces initial injury, and subsequent damage is linked to inflammatory process and to changes in the excitation-contraction coupling within the muscles. Classically myofibrillar ultrastrctural changes in DOMS muscles are mainly related with myofibrillar Z-disc. Z-disc streaming and broadening have long been deemed as the hallmarks of DOMS muscles. Recent studies on rabbit models have shown a rapid loss of the intermediate filament protein desmin after eccentric contractions and this was believed to be the initial event which triggers a subsequent muscle fibre necrosis. Even though numerous studies have been conducted on both human muscles and animal models following eccentric exercise, the mechanisms responsible for the perception of DOMS have not been clearly identified.

To re-evaluate the exercise-induced muscle soreness with respect to the muscle fibre structural changes, in the present study three different modes of eccentric exercise were used as a model to introduce DOMS in healthy young subjects. Biopsies from the soleus muscle and vastus lateralis muscle were taken from control subjects and those who had taken part in the exercise, at different time intervals after exercise. The biopsies were analyzed with general histology, enzymehistochemistry, immunohistochemistry and electronmicroscopy.

All the three exercise protocols induced DOMS, which reached its peak value at 24-48 hour post exercise. Examination of the biopsies taken after the three exercise modes showed no loss of desmin or fibre necrosis of any biopsy. However, in biopsies taken 1 hour post exercise, some influx of fibrinogen into muscle fibres was observed. Despite that, the sarcolemma integrity revealed by stainings with dystrophin and laminin was seemingly not destroyed. Further analysis of the biopsies taken after the downstairs running with high-resolution immunohistochemistry revealed the following alterations: 1) F-actin and desmin were in much greater amounts and distributed differently from normal muscle; 2) alpha-actinin, nebulin and titin were initially lacking in focal areas and were subsequently reappearing. These changes were mainly observed in the 2-3 days and 7-8 days post exercise biopsies. The staining patterns were proposed to represent different stages of sarcomere formation. These findings therefore support the suggestion that myofibrils in muscles subjected to eccentric contractions adapt to unaccustomed activity by the addition of new sarcomeres. Electronmicroscopy showed ultrastructural changes also mainly in biopsies taken 2-3 days and 7-8 days post exercise. These changes were classified into four types on bases of their different staining patterns. For each of the four types of changes, there was a corresponding type of changes revealed by the immunohistochemical method. It was concluded that alterations revealed by electronmicroscopy were suggestive of myofibrillar remodeling rather than the conventionally suggested injury.

The present study will change the dogma that myofibrillar disruption/damage is a hallmark of DOMS. The findings of this study is of clinical importance as the myofibrils contrary to becoming weakened, are reinforced by cytoskeletal elements during the addition of new sarcomeres. The latter gives for the first time a mechanistic explanation for the lack of further damage upon additional exercise (second bout effect). Furthermore, the current methods of analysis of biopsies from eccentric exercised subjects can be used as an in situ model to analyze the molecular changes taking place in the muscle fibres affected by DOMS.

Key words: Eccentric exercise, Human muscles, DOMS, Injury, Z-disc streaming, Intermediate filaments, Z-disc related proteins, Myofibrillar remodelling, Addition of new sarcomeres

ABRBREVIATIONS

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
COX	Cytochrome *c* oxidase
CK	Creatine kinase
DOMS	Delayed onset muscle soreness
FITC	Fluorescein iso-thiocyanate
GA	Glutaraldehyde
IF	Intermediate filament
NADH-TR	Nicotinamide dinucleotide tetrazolium reductase
PAP	Peroxidase-anti-peroxidase
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
SDH	Succinate dehydrogenase
TRITC	Tetraethyl rhodamine iso-thiocyanate

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Yu J-G, Malm C and Thornell L-E.**
Eccentric contractions leading to DOMS do not cause loss of desmin nor fibre necrosis in human muscle.
Histochemistry and Cell Biology 2002; 118 (1): 29-34

II. **Yu J-G and Thornell L-E.**
Evidence of transient increased permeability of sarcolemma upon eccentric exercise inducing delayed onset muscle soreness. *Manuscript*

III. **Yu J-G and Thornell L-E.**
Desmin and actin alterations in human muscles affected by delayed onset muscle soreness: a high resolution immunocytochemical study.
Histochemistry and Cell Biology 2002; 118 (2): 171-179

IV. **Yu J-G, Fürst D. O. and Thornell L-E.**
The mode of myofibril remodelling in human skeletal muscle affected by DOMS induced by eccentric contractions.
Histochemistry and Cell Biology 2003; 119 (5): 383-393

V. **Yu J-G and Thornell L-E.**
Ultrastructural alterations in human muscles with DOMS revisited: Evidence for myofibril remodeling as opposed to myofibril damage. *Manuscript*

INTRODUCTION

Delayed onset muscle soreness

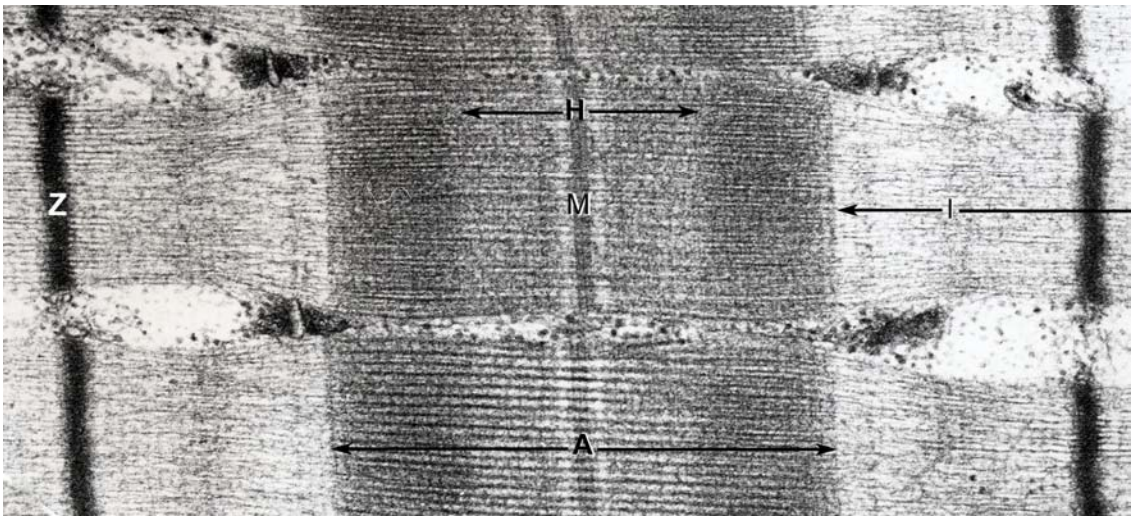
When people perform unaccustomed exercise, particularly if the exercise involves a large amount of eccentric (muscle lengthening) contractions, they often get pain, stiffness, swelling and tenderness in the muscles. The symptoms usually appear a couple of hours to a day, peak between 1 and 3 days, and disappear within 5 to 7 days, after the exercise (Clarkson & Hubal, 2002). This constellation of symptoms is often called "delayed onset muscle soreness (DOMS)".

In 1902, Hough (1902) published the first report on muscle soreness. He suggested that soreness experienced in the finger flexor muscles, 8-10 h after performing rhythmical exercise, was most likely due to "some sort of rupture within the muscle." In the early 1980's, with the advent of more sophisticated techniques, morphological alterations were observed and were interpreted to indicate myofibrillar disruption (Friden et al., 1981; Friden et al., 1983; Newham et al., 1983). These findings stimulated a renewal interest in DOMS and during the past two decades, a considerable amount of information has been obtained on this topic and the prevailing hypothesis is that the myofibril structure becomes ruptured. However the mechanism underlying DOMS still remains unclear (Warren et al., 1999; Clarkson & Hubal, 2002; Lieber & Friden, 2002).

Skeletal muscle structure and functions

Skeletal muscles are composed of muscle fibres which are long cylindrical multi-nucleated cells, 10-100 microns in diameter and often several centimeters long. The entire fibre is surrounded by a basement membrane which when combined with the fibre plasma membrane is histochemically termed the sarcolemma. The bulk of the muscle fibre consists of myofibrils which are the organelles responsible for the production of active force in striated muscles. Each myofibril contains repetitive contractile units called sarcomeres. Under light or electron microscopy, an individual sarcomere consists of a dark central band (A-band) 1.5-1.6 μm long, flanked by two paler bands (I-bands), the lengths of which vary with the state of shortening of the myofibre. The more dense central "A" band is crossed at its mid-point by a dark, narrow transverse line, the "M" line or band, bordered by a paler band of variable width, the "H" zone (Fig. 1a). The A-band is composed of a regular hexagonal array of filaments 15-18 nm in diameter, the principle constituent of which is the protein myosin. The I-bands on either side of the A-band are divided at their midpoints by a narrow dense line, the "Z" line or disc. The I-band is constructed of parallel filaments of paired alpha-helices of chains of a globular protein of actin, in combination with a second globular protein, troponin, and a long-chain protein, tropomyosin. At the Z-disc the I-filaments of the two halves of one I-band form regularly arranged lattices and are cross-linked by alpha-actinin, the major component of the Z-disc. The Z-disc thus marks the longitudinal boundaries of the individual sarcomere, which each consist of one A-band and two half I-bands (Fig. 1b). The free ends of the I-filaments interdigitate between the elements of the hexagonal A-band lattice, in such a manner that each I-filament occupies the center of a triangular space between three adjacent A filaments.

In skeletal muscles, there also exists a cytoskeletal lattice, which can be subdivided into the extra-sarcomeric, the intra-sarcomeric and the subsarcolemmal cyto-skeleton (Fig. 2). The extra-sarcomeric cytoskeleton is comprised of a network of intermediate filaments (IFs), localizing between the myofibrils at the level of the Z-discs, between peripheral myofibrils to the sarcolemma, and between the nuclear membrane to the myofibrils and the sarcolemma. The intermediate filaments are so named because their diameter (8-10 nm) is intermediate between that of the thick (myosin, 15 nm) and the thin (actin, 6 nm) filaments (Ishikawa et al., 1968). Desmin is the main IF protein expressed in mature skeletal muscles.



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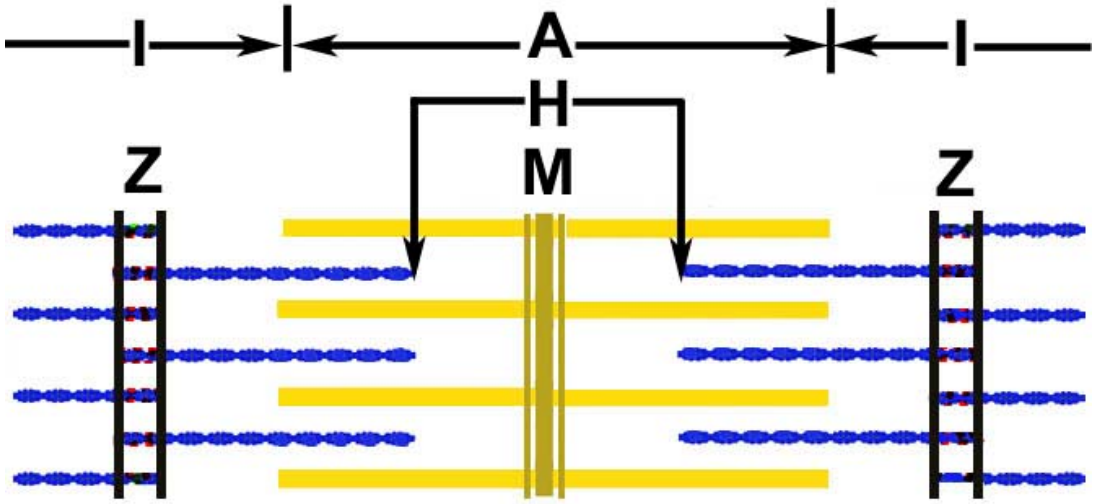

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Fig. 1 (**a**) Longitudinal section of human myofibre including parts of four parallel myofibrils. Dark A bands (A) alternate with pale I bands (I), the latter being bisected at their mid-points by dense Z-discs (Z).The thick filaments of A band possess a set of fine transverse cross-striations at their centres, the M line (M). The thin filaments of the I bands interdigitate with those of the A band, and their central ends delimite the H zone (H). Note the triads are marked with arrows. (**b**) A diagram of the structure of the sarcomere.

The general function of the extra-sarcomeric cytoskeleton is to maintain cell shape and withstand mechanical stress. Nevertheless, it is highly flexible and is involved in a number of other important cell functions like signal transduction and cell movements (Clark et al., 2002). The intra-sarcomeric cytoskeleton consists mainly of titin and nebulin. Titin is a single molecule (the largest vertebrate protein identified to date), which spans half the sarcomeres from the Z-disc to the M-band and it is believed to function as the organizer of the sarcomeres by providing specific, spatially defined binding sites for

other sarcomeric proteins (Trinick, 1996; Maruyama, 1997). Nebulin is also a giant protein, which spans the length of the actin filaments and forms the fourth filament system in skeletal muscle. Nebulin is considered to be associated with actin filaments to serve as a molecular ruler either by restricting the lengths of the actin filaments or by stabilizing the actin filaments at a uniform length (Wang & Wright, 1988; Kruger et al., 1991). The subsarcolemmal cytoskeleton includes membrane and membrane-associated proteins, such as vinculin, spectrin, dystrophin, transmembrane integrins, ankyrin, alpha-actinin and desmin (Price, 1991; Small et al., 1992; Carlsson & Thornell, 2001). These proteins indirectly connect the most peripheral myofibrils with the extracellular matrix in specialized sarcolemmal domains, the costameres (Pardo et al., 1983).

Other components of the muscle fibre are the mitochondria, important for providing energy and the sarcotubular system, responsible for the release and uptake of Ca^{++}, thereby regulating the muscle fibre contraction and relaxation.

The sliding-filament theory of contraction proposes that a muscle shortens or lengthens because the thick and thin filaments slide past each other without changing length. The molecular motor to drive this contraction process is the action of the myosin crossbridges, which cyclically bind, rotate, and detach from the actin filaments with energy provided by ATP hydrolysis. This causes a major change in the relative size of various zones and bands within a sarcomere, and produces a force at the Z-disc. The force generated can be

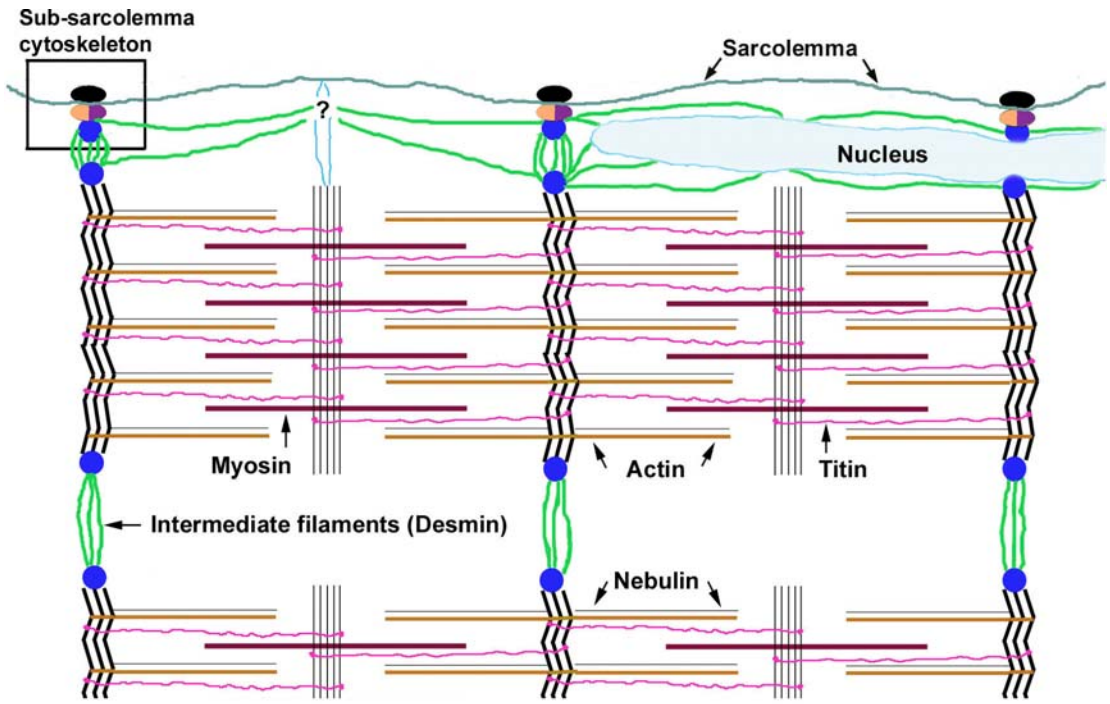

Fig. 2 Schematic drawing of the skeletal muscle cytoskeleton. Two myofibrils composed of sarcomeres, the smallest contractile unit, are shown. The sarcomere is composed of thick myosin and interdigitating thin filaments, and an intra-sarcomeric cytoskeleton, which is made up by titin and nebulin. The extra-sarcomeric cytoskeleton consists of intermediate filaments (IFs), mainly composed of desmin. The sub-sarcolemmal cytoskeleton includes membrane and membrane-associated proteins, such as vinculin, spectrin, dystrophin, transmembrane integrins, ankyrin, alpha-actinin and desmin.

transmitted longitudinally to the tendon and laterally to the membrane, thus also involving the intracellular and extracellular cytoskeleton lattice.

During an eccentric action, a contracted muscle is forced to elongate while producing tension. Its counterpart, concentric action, produces tension during muscle shortening. The intermediate, isometric contraction, produces tension while the muscle remains essentially at the same length.

Hypotheses for DOMS

When my study started in 1997, the prevailing theory of the causes and development of DOMS and subsequent recuperation was as expressed in Figure 3 in a well-known text book of "Exercise physiology" (McArdle et al., 1996).

The flow chart below is of course a summary and approximation of many investigations based on both human and animal studies. When examining the literature it was obvious that there was no definitive evidence to confirm in which order the events occur (Clarkson and Hubal, 2002). Does the sarcolemma become damaged first? Is the initial damage related to the damage of the contractile and noncontractile structures? Is Ca^{++} accumulation an initial event which triggers subsequent steps? Inflammation was at that time thought to be a late phenomenon which initiates the healing process. The scheme also brings up another still unsettled question that DOMS muscle is more resistant to damage from subsequent exercise – the so called second bout effect (McHugh, 2003).

DOMS is undoubtedly associated with the eccentric components of exercise. The tension generated during eccentric contraction is much higher than that of the concentric contraction and is believed to induce a damage on sarcolemma (Enoka, 1996). The increased levels in the blood of muscle fibre proteins [creatine kinase (CK), myoglobin and myosin heavy chain fragments] definitely indicate a loss of cell membrane integrity in subjects with DOMS (Armstrong et al., 1991; McNeil & Khakee, 1992; Clarkson & Hubal, 2002).

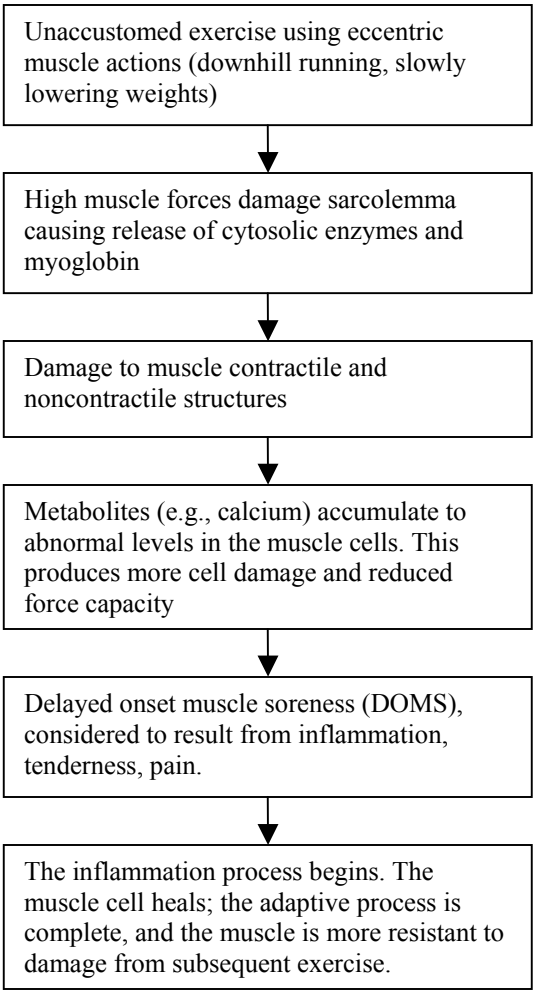

Fig. 3 Proposed sequence for the development of DOMS following unaccustomed eccentric exercise.

An alternative way of examining membrane leakage is to examine if plasma proteins have entered into the muscle fibres. Kent (1966) was the first to point out this possibility. We have evaluated this method in our laboratory and have shown that staining for plasma fibronectin is an excellent marker for irreversibly damage to cardiomyocytes (Thornell et al., 1992; Holmbom, 1997). Staining for plasma fibronectin was also used to study the effects of an exhausting ultra-marathon race, the Western State 100 mile (160 km) (Crenshaw et al., 1993). TC scans of subjects after the race showed increased uptake of technetium pyrophosphate, suggesting considerable muscle necrosis. In contrast, biopsies taken one day after the run

in the most necrotic part of the muscle revealed only 1 % of fibres were necrotic on the basis of morphological criteria. Necrotic fibres staining positively for plasma fibronectin, lacked staining for phosphorylase, had no plasma membranes and were infiltrated with macrophages. Thus there was a large discrepancy between the two methods to evaluate muscle fibre necrosis. However, the morphological one is more accurate (Crenshaw et al., 1993) and has so far not been used on human samples from subjects with DOMS.

In 1981, Friden et al. (1981) published the first morphological study of muscles from people with DOMS. Human soleus muscle biopsies taken 2 and 7 days after the subjects had ran downstairs 10 flights of stairs for ten times were examined. Subcellular abnormalities with frequent focal disturbances of the cross-striated band pattern were seen in the muscle fibres. The abnormalities originated from the myofibrillar Z-disc, which showed a marked broadening, streaming or total disruption. The myo-filamentous material in sarcomeres adjacent to the affected Z-disc was either supercontracted or disorganized and out of register. The interpretation of the findings was that the high myofibrillar tension developed during activation of the contractile material, i.e. the interdigitating arrays of thin and thick myofilaments, had resulted in some mechanical disruption of the Z-disc and that the Z-disc during overloading constituted a weak link in the myofibrillar contractile chain. In a subsequent study, Friden et al. (1983) examined human vastus lateralis muscles taken within 1 h, 3 and 7 days after a backwards cycling exercise. They found that 32%, 52%, and 12%, respectively, of the observed fibres showed evidence of focal disturbance of a similar appearance to that seen in the soleus muscles. This study gave further evidence that DOMS and myofibrillar damage were associated.

The Z-disc related changes like Z-disc streaming, smearing and broadening have since then been observed in many other studies, where different exercise protocols were used on different muscles (see Table 1). Since the Z-disc related changes have been widely observed in muscles with DOMS, they are nowadays deemed as the morphological hallmarks of muscles with DOMS and have been interpreted to represent muscle damage (Lieber et al., 1991; Lieber & Friden, 1993; Warren et al., 1993; Brooks et al., 1995; Hasselman et al., 1995; Jones et al., 1997; Takekura et al., 2001).

In 1984, Friden et al. (1984) published the first observation of desmin intermediate filaments in biopsies from subjects who performed eccentric exercise. In biopsies taken at 3 days post exercise, some longitudinal extensions of desmin were observed. Different hypotheses were proposed for the presence of these longitudinal strands: 1) a mechanical disturbance of the desmin filaments caused by the high tensions developed during eccentric contractions, 2) distension of the cytoskeleton caused by edema, or 3) a cytoskeletal disturbance could be a secondary response to extensive myofibrillar lesions and could reflect sarcomerogenesis. The desmin filaments would thereby act as mechanical integrators for the repair of the filaments. In a later study Crenshaw et al. (1994) reported a partial loss of titin staining in areas of highly variable sarcomere length in biopsies taken 2 days after eccentric exercise. Ultrastructural analysis of the same biopsies revealed multipile hypercontraction zones. Between these zones widened sarcomeres with displaced A-bands were observed. The high tension was suggested to cause fragmentation of titin and the resulting instability would lead to an asymmetric movement of A-bands.

In a recent review Lieber and Friden (2001) suggested that the following series of events take place in muscle fibres after DOMS-causing exercise: cytoskeletal disruption, loss of myofibrillar register, i.e. Z-disc streaming and A-band disorganization, loss of cell integrity as manifested by intracellular plasma fibronectin stain, hypercontraction of injured fibre regions and invasion of inflammatory cells.

Table 1. Summery of morphological studies on human muscles with DOMS

Exercise model	Biopsy	Biopsy time	Major changes
Running downstairs (Friden et al., 1981)	S	Before, 2d, 7 d	Z-band streaming, broadening and, at places, total disruption
30 min EC on cycle ergometer (Friden et al., 1983)	VL	Control, 1 h, 3d, 6 d	Disorganized sarcomere, and Z-band streaming, broadening and, at places, total disruption were observed in 31%, 52% and 12% of the fibres in the biopsies taken at 1 h, 3 days and 7 days post-exercise, respectively.
EC on cycle ergometer (4 or 8 weeks) (Friden et al., 1983)	VL	control, 1 h, 3 d	Small and round fibres and type 2C fibres were seen in the biopsies of 8 weeks training group. Z-band streaming and destroyed sarcomere registrations were seen under electron microscopy.
EC on cycle ergometer (8 weeks) (Friden, 1984)	VL	Control, 3 d	Distorted sarcomere, extension of Z-band material into I-band, Z-band smearing, disturbed Z-band alignment, separated Z-band material and bisected Z-band were observed.
EC cycle ergometer (Friden et al., 1984)	VL	1h, 3d, 6d	At 3 days post exercise, desmin single strands were seen.
Step-test (Newham et al., 1983)	QF	Before, 0, 24-48 h	Disorganized myofilaments; Z-line streaming; loss of Z-line
Arm-curl exercise Walking downhill (Jones et al., 1986)	BB, G	2-20 d	Little or no change in the first 7 days after the exercise but later degenerating fibres were seen, as well as infiltration by mononuclear cells and eventually, by 20 days, signs of regeneration. In the severely affected calf muscle type II fibres were preferentially damaged
Arm-curl exercise Walking downhill (Round et al., 1987)	BB, G	Control, 4-20 d	Mild damage in biopsies taken 4 and 5 days post exercise but this become more severe with time and there still signs of damage at 14 days. Damage included necrotic fibres, increased acid phosphatase activity and invasion of tissue with mononuclear cells
EC on cycle ergometer (O'Reilly et al., 1987)	VL	Before, 0 h, 10 d	More type I fibres were glycogen depletion; numerous ultrastructural abnormalities were seen in 0 h biopsies; in 10 d biopsies, necrotic fibres were seen.
400 EC (Friden et al., 1988)	TA	48 h	Overall morphology of the specimens revealed a greater cross-sectional fiber area (both type 1 and type 2) in the eccentrically exercised muscle. Inflammation was only seen in 1 of 8 of the "eccentric" muscle samples and no fiber necrosis was observed. Extremely large type 2 fibers were found in 4 of 8 subjects from the eccentric specimens.
70 maximal EC (Stauber et al., 1990)	BB	48 h	Mast cell degranulation, separation of extracellular matrix from myofiber, increased plasma constituents in the extracellular space
70 maximal EC (Stauber et al., 1991)	BB	48 h	Labeled antibody against proteoglycan revealed extracellular matrix torn away
EC on cycle ergometer (Manfredi et al., 1991)	VI	Before, 0 h, 10 d	In the older subjects, 90% of the post exercise fibres showed focal damage, whereas in the young subjects, the value was 5 to 50%. The damage was seen as Z-band streaming and smearing; loss of I-, A-, and Z-bands

30 min downhill running (Nurenberg et al., 1992)	S,G, TA, PL	48 h	9 subjects have increased signal intensity (SI) of MR in multiple muscles, whereas 6 subjects have an increase of SI in only one muscle and 3 subjects have no increase in SI. Injury was seen as Z-band streaming and loss of sarcomere.
45 min downhill running (Fielding et al., 1993)	VL	Before, 45 min, 5 d	45 min after exercise, 32.5% of the total Z-disc volume was damaged, whereas 5 days afterwards, that value decreased to 14.1%. The damage was mainly Z-band related.
160 km footrace (mostly downhill) (Crenshaw et al., 1993)	G	24 h	1% of the 3698 fibres analysed were necrotic and fibronectin staining positive; titin irregular in longitudinal section, widened Z-disc and amorphous Z-disc material extending into adjacent I-bands or A-bands.
Knee extension (Crenshaw et al., 1994)	VL	2 d	Z-disc streaming and Z-disc smearing; thick filament displacement to one side of the sarcomere. The changes occurred predominantly in type 2 fibres.

EC: Eccentric contraction. BB: Biceps Brachii. QF: Quadriceps femoris. S: Soleus. VL: Vastus lateralis. TA: Tibialis anterior. G: Gastrocnemius. PL: Peroneus longus.

Animal models used to mimic human muscle eccentric contractions

One large problem in studying human muscles with DOMS is that it is hard to manipulate the muscle lengths and strengths and to correlate their changes with muscle injury and DOMS. Furthermore, it is ethically difficult to perform many repetitive biopsies. Armstrong et al. (1983) stated in 1983 "while there is no proof that morphological damage in rodent muscle and delayed muscle soreness in human subjects are manifestations of the same exercise-induced cellular events, it is reasonable to suspect they are." For these reasons many animal models have been used to study the injury caused by eccentric contractions. Briefly in the past two decades, three different animal models were used: 1) free running intact animals (Armstrong et al., 1983; Ogilvie et al., 1988; Duan et al., 1990; Komulainen et al., 1994); 2) experimental set-ups with unconscious animals (McCully & Faulkner, 1985; McCully & Faulkner, 1986; Stauber et al., 1988; Brooks & Faulkner, 1990; Zerba et al., 1990; Lieber et al., 1991; Lieber & Friden, 1993; Warren et al., 1993; Brooks et al., 1995; Mishra et al., 1995; Lieber et al., 1996) and 3) isolated muscle preparations from animals (Julian & Morgan, 1979; Morgan, 1990; Macpherson et al., 1996; Talbot & Morgan, 1996; Jones et al., 1997; Talbot & Morgan, 1998).

The investigations of Armstrong and his colleagues are an example of the first category (Armstrong et al., 1983; Ogilvie et al., 1988). They investigated rats, which were forced to run downhill on a treadmill and observed disruption of myofibrillar banding patterns, accumulation of macrophages, mononuclear cell infiltration and necrotic fibres (Armstrong et al., 1983). Similar results have also been reported by other researchers using downhill running animal models (Duan et al., 1990; Komulainen et al., 1994). Notably, in these animal studies muscle damage almost always develops progressively, producing minor ultra-structural changes like Z-disc streaming and smearing to muscle fibre necrosis and inflammation (Armstrong et al., 1983; Ogilvie et al., 1988; Duan et al., 1990; Komulainen et al., 1994). Komulainen et al. (1994) demonstrated that different parameters of injury following lengthening contractions could present different time courses. In rat muscles following downhill running, broadened Z-discs and neutrophil infiltration appeared earlier than necrosis and macrophage invasion, and broadened sub-sarcolemmal area.

The studies of Lieber and Friden are an example of the second category (Lieber et al., 1991; Lieber & Friden, 1993; Lieber et al., 1994; Lieber et al., 1996; Friden & Lieber, 1998). They carefully designed a rabbit model to mimic human muscle eccentric contractions (Lieber et al., 1991). Using this model they found that muscles actively strained 12.5% beyond resting length experienced a 40% decrease in maximum tetanic tension. Muscles actively strained 25% beyond resting length experienced a 60% decrease in maximum tetanic tension. Thus, muscle damage was a function of the length to which the muscle was elongated during stimulation rather than the magnitude of the contractile stimulus (Lieber et al., 1991; Lieber & Friden, 1993).

Using this rabbit model, Lieber, Thornell and Friden (1996) observed a very rapid loss of staining for desmin in muscle fibres after 5-15 min of eccentric contractions. This did not occur after either isometric or concentric contractions. They speculated that the desmin loss pointed to some type of enzymatic hydrolysis or protein phosphorylation as a likely mechanism rather than gene regulation, which would require much more time. Furthermore they noticed that most fibres, which did not stain for desmin, stained for plasma fibronectin, indicating the loss of muscle fibre membrane integrity of these fibres. Notably some fibres lacked desmin but did not contain plasma fibronectin, suggesting that the desmin loss was prior to the membrane damage. The muscle fibres in this model developed into segmental necrosis and inflammation, followed by repair and regeneration (Friden & Lieber, 1998).

The "Popping-sarcomere hypothesis" is the result of studies related to the third category of experiments. The rationale of the hypothesis is as follows (Fig. 4) (Proske & Morgan, 2001).

During the active stretching of a muscle, most of the length change will be taken up by the weakest sarcomeres. On the descending limb of the length-tension curve, these sarcomeres will become progressively weaker and when they reach their yield point they will lengthen rapidly to a point of no myofilament overlap. This process is repeated iteratively, with the next weakest sarcomere stretching, and so on. When the muscle relaxes, these overstretched sarcomeres presumably mostly reinterdigitate. However, a few may fail to do so and become disrupted (Morgan, 1990; Proske & Morgan, 2001). During repeated eccentric contractions it is postulated that the number of disrupted sarcomeres grows, until a point is reached where membrane damage occurs. It is at this point that damage to elements of E-C coupling machinery becomes apparent. Subsequently the fibre may die (Proske & Morgan, 2001). In the flow diagram below the disruption of sarcomeres precedes membrane damage and necrosis.

Several reviews on eccentric exercise-induced injury have recently been written by Lieber and Friden (Lieber & Friden, 1999; Lieber et al., 2002; Lieber & Friden, 2002). In one of these, "Mechanisms of muscle injury gleaned from animal models" (Lieber & Friden, 2002), they have incorporated all of the current data (discussed above). They suggested that the earliest events associated with injury are mechanical in nature. Muscle

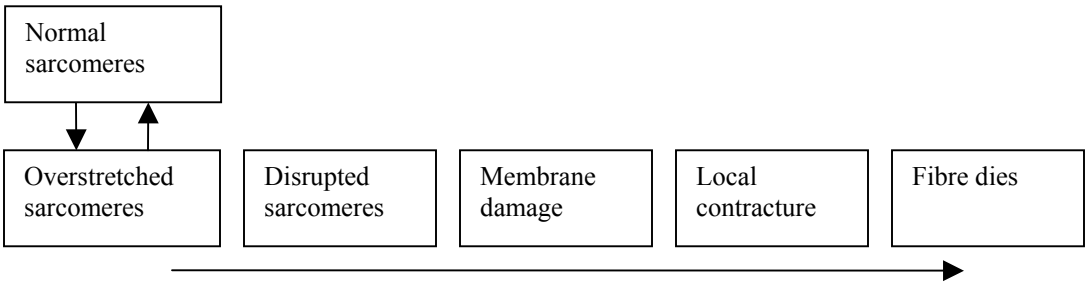

Fig. 4 Postulated series of events leading to muscle damage from eccentric exercise.

fibre strain results in an increased [Ca^{++}]. Such an increase may be due to calcium influx via strain-activated channels, by disruption of the intracellular stores of calcium in the sarcoplasmic reticulum, or by disruption of the T-system or sarcolemma. This may be related to the concept of sarcomere "popping" that has been proposed as a damage mechanism during eccentric contraction. After the increased [Ca^{++}], calpain activation results in selective hydrolysis or disruption of the intermediate filament network. Finally, after the intermediate filament network has been altered due to protolysis or conformational changes, the myofibrillar apparatus is disrupted during repeated muscle activation and is unable to develop normal tension. Inflammation that occurs after injury further degrades the tissue.

Experimental evidence for the proposed hypothesis to be true also for human muscles affected by eccentric exercise is however lacking. Furthermore, none of the hypotheses based on animal models can explain "the second bout effects", i.e. a repeated bout of similar eccentrically biased exercise results in markedly reduced symptoms of damage than the initial bout (McHugh, 2003).

AIMS OF THE PRESENT STUDY

The aims of the present study was to re-investigate with modern methods the effects of eccentric exercise leading to DOMS on muscle morphology in order to get further insight into the mechanisms of DOMS.

The following specific questions were addressed:

1) Does eccentric exercise induce an injury to the sarcolemma? (I, II)

2) Does eccentric exercise leading to DOMS cause loss of desmin and myofibre necrosis in human muscles? (I)

3) Do cytoskeletal changes in muscles with DOMS indicate muscle damage? (III, IV)

4) Do the morphological hallmarks of DOMS – the Z-disc alterations represent myofibrillar damage? (V)

MATERIALS AND METHODS

Subjects

Thirty-five subjects took part in this study. Ten male subjects with a mean age of 24.3 years (range 21-30 years) participated in a downstairs running exercise protocol (I-V). Seven male subjects with a mean age of 23.9 years (range 19-32 years) participated in eccentric cycling (I) and another five male and one female subjects with a mean age of 25.8 years (range 18-39 years) participated in downhill 8° treadmill running (I). Twelve male subjects who did not take any physical exercise served as controls.

All subjects were informed about the meaning of the study and were asked to refrain from unaccustomed exercise during the experimental period. All subjects signed an informed consent document consistent with the Declaration of Helsinki and the policy of the Ethical Committees at Umeå University or at the Karolinska Institute.

Experimental procedures

The downstairs running exercise protocol performed by the subjects in this study was similar to that used previously (Friden et al., 1981). In brief, the subjects were asked to run downstairs from the 10^{th} floor to the ground floor and then to take the elevator back to the 10^{th} floor and repeat the procedure 15 times. Muscle soreness was evaluated twice daily for 8.5 days successively and the degree of pain was self-estimated on a 0-10 subject rating scale (0 = no soreness and 10 = very, very sore).

For the eccentric bicycling, an electrically powered bicycle was used as described previously (Friden et al., 1983). Subjects were instructed to maintain 60 rpm for 30 min at a work rate equal to the highest concentric cycling work rate maintained for 2 min during the concentric cycling $V_{O2,max}$ test. All subjects performed eccentric cycling at 250 or 300 W and the eccentric exercise can be considered maximal or close to maximal for most subjects, with respect to eccentric muscular exercise capacity. Muscle soreness was self-estimated on the 0-10 rating scale at the time points of blood and muscle sampling (before, immediately after and 6, 24 and 48 h, and 4 and 7 days post-exercise). Blood for CK analysis was drawn from an arm vein into heparinised tubes. For further details see Malm et al. (2000).

Downhill running was conducted on a treadmill. After a 10- to 15-min warm-up at a speed chosen by the subject and a brief resting period (approximately 5 min) the test started on a treadmill with a 1° slope and this was increased 1° every minute. All subjects managed to run at the chosen speed for 45 min. Before, 24, 48 and 72 h, and 7 days after exercise muscle soreness was self-estimated on the 0-10 subject rating scale in the right thigh muscles when the subjects were in a prone, dorsal position with the right leg lifted 5 cm from the surface. Muscle pain was self-evaluated by placing a rubber cylinder (area = 5 cm^2) attached to a 5-kg weight (giving a pressure of 10 N cm^{-2}) on the midsection of the right vastus lateralis muscle. For further details see Malm (2001).

Muscle biopsies

Using local skin anaesthesia, surgical biopsy from the soleus muscle was obtained from both controls and downstairs running exercised subjects. From four subjects one biopsy was taken at 1 hour post-exercise, and from the other six subjects, two biopsies were taken at 2-3 days (one leg) and 7-8 days (the other leg) post-exercise. A control biopsy was taken from the soleus muscle on control subjects. After eccentric bicycling, biopsies from the vastus lateralis were taken from both the controls and the exercised subjects, using the forceps biopsy technique. In both the control and exercise group, the first, second, fourth and sixth biopsies were taken in the left leg and the third, fifth and seventh biopsies were taken in the right leg. Muscle biopsies were taken from the left vastus lateralis 48 h after downhill treadmill running

using the same procedure as in the eccentric cycling protocol.

Each biopsy was divided into two pieces. One was mounted in embedding medium (Tissue-Tek OCT; Miles, Elkkhart, Ill., USA), frozen in propane chilled with liquid N_2 (-160°C) and stored at -80°C until used. These frozen specimens were used for enzyme- and immunohistochemistry. The other piece was slightly stretched and mounted on a piece of cork with pins and then fixed for 2 h with freshly prepared 2% paraformaldehyde (PFA). After washing in phosphate-buffered saline (PBS) buffer twice, the muscle sample was divided into two pieces. One was processed for thin and semi-thin sections and stained with immunohistochemistry and the other one for plastic embedding and sectioned for toluidine blue staining and for transmission electron microscopy.

Histology and enzymehistochemistry (I)

Serial transverse and longitudinal sections (5-8 μm thick) were cut at -25°C on a Reichert Jung cryostat (Leica, Nussloch, Germany). Haematoxylin-eosin and a modified Gomori trichrome stain were used for basic histopathology (Dubowitz, 1985). Myofibrillar adenosine triphosphatase (ATPase) histochemical analysis performed after preincubations at pH 4.3, 4.6 and 10.4 revealed muscle fibre types (Dubowitz, 1985). Nicotinamide dinucleotide tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX) were used for evaluation of mitochondrial activity.

Immunohistochemistry (I-IV)

Muscle samples were cut in small blocks (1 × 3 mm) along the longitudinal axis of the muscle and infiltrated in 10% sucrose over night. Some blocks of the specimens were mounted in embedding medium with the fibres parallel to the surface of a piece of cardboard and rapidly frozen in propane chilled with liquid nitrogen (-160°C). Serial longitudinal cryo-sections (3 μm) were cut at -25°C on a Reichert Jung cryostat (Leica, Nussloch, Germany) and collected on chrome-alun-gelatine coated glass slides. Other blocks of the specimens were further cut in 1 mm^3 cubes and incubated in 2.3 M sucrose for 24 h. The specimens were then put on special stubs with the fibres parallel to the surface and frozen in liquid nitrogen. To obtain semi-thin sections (approximately 0.5 μm) the specimens were sectioned at -75°C with a Reichert Ultracut microtome equipped with a FCS cryo attachment (Leica, Nussloch, Germany). The sections were collected on chrome-alun-gelatine coated glass slides and stained for immunofluorescence analysis.

For this purpose, the sections were washed in 0.01 M PBS and immersed in 5% normal serum (Dako, Glostrop, Denmark). Excess serum was wiped off and sections were incubated with primary antibodies either for 60 min at 37°C or over night at 4°C, in the latter with a ten-fold dilution of antibodies. In Table 2 all primary antibodies used in the different papers were listed. Detection of the bound antibodies was performed with standard indirect peroxidase-anti-peroxidase (PAP) or fluorescence techniques. Visualization of the antibodies was revealed in a solution containing diaminobenzidine and hydrogen peroxide in the case of PAP staining, whereas a fluorechrome (FITC, green fluorescence; Cy3, red fluorescence; Alexa 488, green

Table 2. Overview of antibodies used

Antibody	Clone	Host	Paper
Alpha-actinin	EA-53	Mouse	IV
Alpha-actinin	653	Rabbit	IV
Alpha-actinin	2A	Rabbit	IV
Desmin	A0611	Rabbit	I, III, IV
Desmin	D33	Mouse	I, III, IV
Dystrophin	Dys1	Mouse	II
Dystrophin	Dys2	Mouse	II
Dystrophin	Dys3	Mouse	II
Dystrophin	5EM96	Rabbit	II
Fibrinogen	A080	Rabbit	II
Fibrinogen	85D4	Mouse	II
Fibronectin	341635F12	Rabbit	I, II
Laminin	M	Mouse	II
Lectin		Rabbit	II
Nebulin	NB2	Mouse	IV
N2.261		Mouse	II
Titin	Z1	Rabbit	IV

fluorescence; Alexa 568, red fluorescence) (Dako or Jackon Immuno Research Laboratory, Pennsylvania, USA) conjugated to the secondary antibodies was used for immunofluorescence. Control sections were treated as above, except that the primary antibody was exchanged with non-immune serum. Sections were observed using a Zeiss (Carl Zeiss, Oberkochen, Germany) microscope and photographed with a 3CCD camera (Dage-MTI Inc., Michigan City, USA) or a Nikon microscope (Eclipse, E800; Tokyo, Japan) equipped with a Spot camera (RT color; Diagnostic Instruments, USA). Micrographs were processed with Adobe Photoshop (Adobe System, mountain View, CA., USA).

Light microscopy and transmission electron microscopy (V)

The muscle tissues were fixed with 2.5% glutaraldehyde (GA) in 0.1 M PBS at pH 7.4 for at least 24-48 h. After washing several times in PBS buffer, the muscle was transversely cut into slices about 1 mm thick. Each slice was then further subdivided into 1 mm^3 blocks and fixed in 1% osmium tetroxide for 2 h. Specimens were dehydrated in a graded series of acetone and embedded in Vestopal W.

Semithin (1 μm) sections were cut on a Reichert Ultracut microtome (Leica, Nussloch, Germany) and stained with toluidine blue. From each biopsy, 3 to 5 randomly selected tissue blocks were cut. The sections contained about 40-50 fibres. Under the light microscope, the whole sections were photographed at low (20-40 times objective lens) magnification. Prints were mounted together to provide an entire view of each of the sections. At higher magnification (100 times objective lens), all muscle fibres in the sections were carefully examined for any irregular band patterns. These were marked on the photographic prints and counted. Ultrathin sections (60 nm) were cut from the specimen blocks and the sections were collected on keyhole Formvar carbon coated grids. The sections were stained with uranyl acetate and lead citrate on a LKB 2168 ultrostainer and observed in a Jeol 1200 EX II electron microscopy (Jeol, Tokyo, Japan).

Data collecting and image analysis

From the thin and semi-thin longitudinal sections stained with desmin, phalloidin, alpha-actinin, titin and nebulin, three types of irregular staining were observed: areas with an increase of staining for desmin and actin, areas with a lack of staining, and areas with supernumerary sarcomeres, for alpha-actinin, titin and nebulin. Transmission electron microscopy revealed four types of changes in myofibre ultrastructure: amorphous widened Z-discs, double Z-discs, amorphous sarcomeres and supernumerary sarcomeres. The number of fibers with any of these changes was counted. If a fiber had more than one of these modifications, the fiber was counted repeatedly. In toluidine blue-stained sections, the percentage of muscle fibres showing any alterations in the transverse band pattern was determined. From the cross sections stained with fibrinogen, the number of fibers with any visible amount of fibrinogen staining within the fiber outlined by laminin or dystrophin staining was counted for each biopsy. For statistical analysis, the number of such fibres from biopsies taken at the same time was pooled together and then compared between groups.

Statistical analysis

The difference in the mean values of self-estimated muscle soreness was compared, before and after exercise, at each time point. The frequencies of the all types of alterations observed at different times after the eccentric exercise were also compared. One-way analysis of variance (ANOVA) and unpaired *t*-test was performed using the StatView 4.5 software (Abacus Concepts, Berkeley, Calif., USA). A probability of rejecting the null hypothesis of less than 0.05 was considered a statistically significant difference. All data are presented as means and standard deviations.

RESULTS

Muscle soreness (I)

All exercise protocols used resulted in DOMS in all subjects and serum CK activity was significantly increased when measured. The peak values of DOMS appeared at 48 h after the exercise for all the three types of exercise and the mean maximal values of the self-estimated muscle soreness varied from 5.2 (downhill running) to 7.8 (downstairs running). After eccentric cycling the highest peaks of CK in the serum were observed at 6 h (166.4 SD 171.8 U/L) and 24 h (155.0 SD 124.3 U/L) post exercise, whereas after downhill running CK in serum was elevated maximally to 936 U/L (SD 146) 24 h post exercise.

Changes in the sarcolemma integrity (I, II)

In the sections stained with anti-plasma fibronectin, strong staining was seen in the endothelial cells of the capillaries and a somewhat weaker staining was observed between the muscle fibres. Large amounts of staining for plasma fibronectin within myofibre as seen in rabbit models following lengthening contractions (Lieber et al., 1996) was not seen in any of these human biopsies. No evidence of muscle fibre degeneration, hyaline fibres or infiltration of mononuclear cells was observed in sections stained using routine histological staining.

When the muscle biopsies were stained with antibodies against fibrinogen, however, some abnormal stainings were seen especially in the 1 hour post exercise biopsies (Table 3). The significant of this finding was stressed by the statistical analysis which revealed that only the value of 1 hour group showed a significant increase compared with that of control samples. Staining for fibrinogen was present within some muscle fibers adjacent to plasmalemma and exhibited a gradient towards the center of muscle fibers. The degree of the staining varied widely from fibre to fibre, ranging from only one minor focal subsarcolemmal site to several focal spots covering almost half of the muscle fibre. In longitudinal sections focal subsarcolemmal stainings of variable lengths along the myofibre were seen and showed a periodic sarcomeric pattern. Double immunostaining with lectin revealed that a capillary was often seen close to this staining. Series sections stained with N2.261 did not show a fibre type specific staining for fibrinogen. None of the two sarcolemmal proteins examined, dystrophin and laminin, showed any abnormality in these post exercise muscles.

Table 3. Number of fibres with intracellular fibrinogen in each group of biopsies (values are mean ± SD expressed as % of all fibres examined in each group)

	Intracellular fibrinogen
Control (n = 6)	3.7 ± 2.4
1 h (n = 4)	15.0 ± 10.8*
2-3 d (n = 6)	5.5 ± 4.7
7-8 d (n = 6)	4.3 ± 4.8

* Significantly different at $P < 0.05$ compared with control value

Changes in the muscle cytoskeletal proteins and myofibril proteins (I, III, IV)

In frozen cross sections, all muscle fibres were stained with antibodies against desmin. However, in some sections of the post-exercise biopsies, the desmin staining sometimes formed an irregular network or showed highly stained dots. In frozen longitudinal sections, many irregularities such as variability in spacing between the striations, focally increased staining and longitudinal strands of staining were apparent.

When muscle biopsies were analyzed with high-resolution immunohistochemistry using antibodies against desmin, alpha-actinin, titin, nebulin, and a ligand for actin, control biopsies as well as unaffected fibres from post-exercise biopsies gave typical cross-striated patterns. Anti-desmin antibody,

Table 4. Alterations of staining for desmin and phalloidin in control biopsies and exercised biopsies taken at different times post exercise (values are mean ± SD expressed as % of all fibres examined in each group)

	Desmin longitudinal strands	Increase of desmin	Increase of phalloidin
Control (n = 6)	4.6 ± 3.3	2.0 ± 2.2	2.2 ± 1.9
1 h (n = 4)	5.0 ± 3.2	1.4 ± 1.8	2.4 ± 2.3
2-3 d (n = 6)	3.9 ± 3.4	8.7 ± 3.9*	12.5 ± 5.8*
7-8 d (n = 6)	5.8 ± 4.5	11.4 ± 4.6*	6.1 ± 2.3*#

* Significantly different from control and 1 h post exercise groups
# Significantly different from 2-3 d post exercise group

however, also stained longitudinal strands, which typically extended from one Z-disc to another Z-disc. Similar longitudinal strands were not seen in staining with alpha-actinin, titin, nebulin, or with ligand phalloidin. The relative number of fibres with longitudinal desmin strands did not show significant difference be tween the groups (Table 4).

In the biopsies taken 2-3 and 7-8 days post exercise, areas with varying degrees of morphological alterations were revealed for all the of stainings. The lesions could be divided essentially into three groups (Fig. 5): (1) areas with an increased staining for desmin and phalloidin; (2) areas devoid of staining for titin, nebulin and alpha-actinin; (3) myofibrillar areas of variable width strongly stained for desmin and phalloidin and revealing supernumerary sarcomeres stained for alpha–actinin, titin and nebulin. The results of the different staining patterns are summarized in Tables 4 and 5. Accordingly, at both 2–3 days and 7–8 days after exercise the number of fibres with an increased staining for desmin and phalloidin and the number of fibres with an absence of staining for alpha-actinin, titin and nebulin were significantly increased relative to controls and to the 1 h after exercise group, respectively (mean values of the latter two groups of samples were statistically similar). With regard to the areas showing super-numerary sarcomeres, most areas were seen in the samples from the 7–8 day group but were not statistically different from that of the 2–3 day group. On the other hand these values were significantly higher than those of the control group and the 1 h after exercise group (mean values of the latter two groups of samples were statistically similar).

In areas of increased staining for desmin, phalloidin always displayed an increased staining. At higher magnifica-tion the strong desmin staining was seen as many single strands in parallel and of various lengths which in general terminated at the level of a Z-disc. In areas with increased phalloidin staining, staining for desmin was either increased or the banding pattern was partly disorganized, being partly displaced and connected by some desmin-stained longi-tudinal strands.

Together with the increased staining for desmin and F-actin, staining for alpha-actinin, titin and nebulin revealed two types of alterations: lack of staining and supernumerary sarcomeres. Additionally, in areas with an increased staining for F-actin, occasional transverse broad bands were seen, where desmin, alpha-actinin and nebulin staining formed two parallel lines around the broad bands. It is noteworthy that although alpha-actinin, titin and nebulin were lacking, the general myofibrillar organization was still preserved and no contraction bands or overstretched sarcomeres were observed in the regions.

The areas exhibiting supernumerary sarcomeres varied in size from only two sarcomeres within one myofibril to over twenty successive sarcomeres spanning half of the diameter of the fibre. In these areas the number of cross-striations revealed by the antibodies against alpha-actinin, titin and nebulin were greater than the number in adjacent myofibrils. Of special interest were the areas where desmin staining was delta-shaped and where the three stainings revealed extra sarcomeres inserted into the pre-

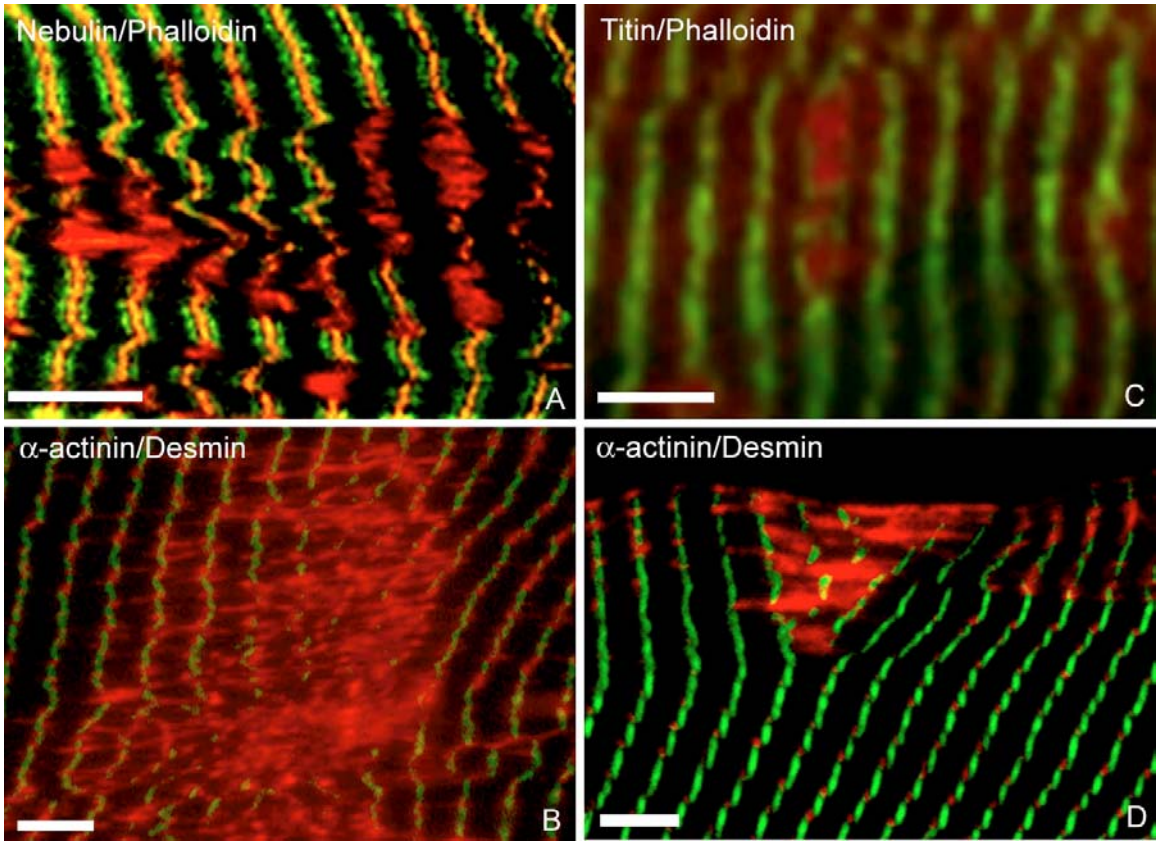

Fig. 5. Double immunolabelling of longitudinally sectioned biopsies taken 2-3 days (**A, B**) and 7-8 days (**C, D**) post-exercise. **A** Phalloidin stained broad bands at the level of Z-discs where nebulin staining was absent. **B** Lack of staining for alpha-actinin in an area with increased staining for desmin. **C** Titin is localized at the edges of the phalloidin-stained broad band. **D** Extra sarcomeres revealed with staining for alpha-actinin in an area strongly stained for desmin.

Table 5. Alterations of staining for α-actinin, titin and nebulin in control biopsies and exercise biopsies taken at different times post exercise (values are mean ± SD expressed as % of all fibres examined in each group)

	Lack of staining			Supernumerary sarcomere		
	α-actinin	titin	nebulin	α-actinin	titin	nebulin
Control (n = 6)	1.4 ± 2.5	1.4 ± 1.4	2.1 ± 1.7	1.6 ± 1.2	3.3 ± 2.0	0.8 ± 1.7
1 h (n = 4)	2.3 ± 2.0	0.6 ± 1.0	1.6 ± 2.0	2.6 ± 3.4	1.5 ± 2.6	2.8 ± 2.0
2-3 d (n = 6)	11.6 ± 4.3*	12.4 ± 5.8*	8.9 ± 3.6*	9.4 ± 5.7*	8.4 ± 3.4*	8.5 ± 4.0*
7-8 d (n = 6)	6.6 ± 3.1*#	7.3 ± 4.1*	5.8 ± 2.2*	1 2.5 ± 7.2*	10.6 ± 5.2*	12.3 ± 6.8*

* Significantly different from control and 1 h post exercise groups
\# Significantly different from 2-3 d post exercise group

existing myofibrils. In general the number of dots and lines stained for alpha-actinin coincided with the number of dots and lines stained for titin. By comparing the intensity of staining of the pairs of lines stained with the nebulin antibody related to each Z-disc, it was obvious that the line facing the outer sarcomeres of each delta-shaped area was

more strongly stained than the inner line. This indicates that the outer sarcomeres of the delta-shaped area are more mature than the inner ones.

Changes in the myofibril ultrastructure (V)

In biopsies from both controls and exercised persons, the myofibrils showed a very regular banding pattern in the toulidine blue stained longitudinal semi-thin sections. Nevertheless, focal alterations to a varying degree, involving one to more than ten sarcomeres in length and one single myofibril to the whole fibre in width, were seen. Often the sarcomeres were out of register in areas with focal alterations or when supernumerary sarcomeres were present. The number of fibres with myofibrillar alterations varied considerably between groups (Table 6).

Using electron microscopy, the focal disturbances of the myofibrillar banding pattern were readily apparent and could be divided essentially into four types (Fig. 6): (1) amorphous widened Z-discs; (2) amorphous sarcomeres; (3) double Z-discs and (4) supernumerary sarcomeres. The frequency of the different patterns is given in Table 6. Statistical analysis revealed that the relative number of fibres for all the four types of changes was significantly larger in the "2-3 day" group and "7-8 day" group than those of the "control" group and the "1 h" group. In addition the "amorphous widened Z-discs" type was significantly more frequent in the "2-3 day" group than in "7-8 day" group.

"Amorphous widened Z-discs" were alterations in which mainly the Z-disc was affected (Fig. 6A, B). The electron dense Z-disc was in transverse continuity with an area composed of an amorphous broadened band of lower density. The altered band could be twice the size of a normal Z-disc or could extend to the A-I border. The broadened band was often asymmetric. Furthermore, the I-bands, related to the altered Z-disc, were often wider than those of neighboring unaffected sarcomeres. Clusters of dense particles were often present close to the affected Z-discs.

"Amorphous sarcomeres" were characterized by a disturbed sarcomeric pattern of one or more sarcomeres (Fig. 6C). Within the affected sarcomeres, the A-band, the M-band and the I-band could not be distinguished as separate units but appeared rather filamentous or amorphous. Such sarcomeres had an electron density in between that of regular I-bands and Z-discs. The Z-discs in such areas varied in appearance. Some Z-discs appeared partly amorphous and showed a lower density than normal ones, whereas in other areas no structures of Z-disc density were apparent at all.

"Double Z-discs" were alterations consisting of electron dense material of Z-disc density present as double rows of dots or lines at the location of a Z-disc (Fig. 6D).

Table 6. Alterations of myofibril banding patterns in control biopsies and exercise biopsies taken at different times post exercise (values are mean ± SD expressed as % of all fibres examined in each group)

	Light microscopy	Electron microscopy			
		Amorphous widened Z-discs	Double Z-discs	Amorphous sarcomeres	Supernumerary sarcomere
Control (n = 6)	3.2 ± 1.8	1.8 ± 1.0	2.3 ± 0.6	1.9 ± 1.7	3.5 ± 1.6
1 h (n = 4)	5.8 ± 2.3	2.8 ± 1.7	3.0 ± 1.0	3.3 ± 2.4	3.9 ± 2.6
2-3 d (n = 6)	25.3 ± 7.6*	13.3 ± 4.1*	5.3 ± 1.9*	18.1 ± 5.4*	11.0 ± 4.7*
7-8 d (n = 6)	13.1 ± 5.5*#	5.3 ± 1.7*#	8.8 ± 4.1*	13.4 ± 5.5*	16.8 ± 6.5*

Significantly different at $P < 0.05$ compared with:

* respective control and 1 h values; # respective 2-3 d value.

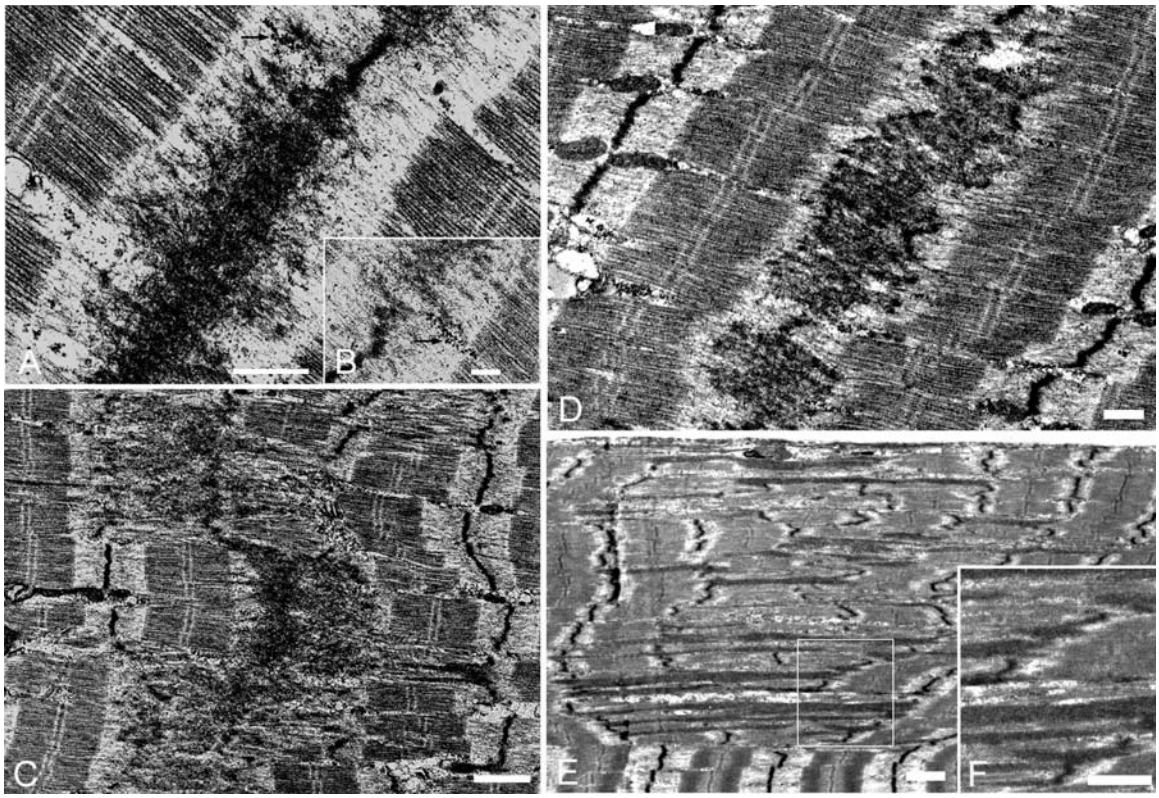

Fig. 6. Electron micrographs of longitudinally sectioned biopsies taken from the exercised subjects at 2-3 days (**A-C**) and 7-8 days (**D-F**) post-exercise. **A, B** Amorphous widened Z-discs. Note the clusters of ribosomes between myoifibrils (arrows). **C** Amorphous sarcomeres. **D** Double Z-discs. **E** Supernumerary sarcomeres, which contain electron-dense longitudinal strands.

The electron dense dots and lines were interconnected by amorphous or filamentous material of lower density than that of the Z-disc but higher than that of the I-bands. In places the filamentous material appeared to contain thick filaments and a small M-band in the middle of the altered segment. This Z-disc alteration was in continuity with the I-bands of the next sarcomeres.

By definition, "supermumerary sarcomere" means that if one follows the Z-discs in transverse register across the myofibrils, one or more additional sarcomeres are present in some myofibrils (Fig. 6E. F). "Supernumerary sarcomere" usually contains dense longitudinal strands, which extend out from the Z-discs and interlink one or more sarcomeres in longitudinal register from Z-disc to Z-disc. The density of the longitudinal strands varied. Most frequently they were less dense than the Z-discs but more dense than the A-bands.

DISCUSSION

Contrary to current theories on causes of DOMS, this thesis shows that eccentric exercise leading to DOMS does not cause loss of desmin and myofibre necrosis in human muscles. Our immunohistochemical studies undoubtedly show an increase of the cytoskeletal protein desmin which is interpreted not to be a sign of damage. Evidence of a temporary injury to the sarcolemma is however given, which is consistent with the leakage of muscle fibre proteins into the blood.

The morphological hallmarks of DOMS, the Z-disc alterations previously thought to represent myofibrillar damage, is here shown to reflect an alteration which leads to myofibrillar remodeling with an increase in the number of sarcomeres within preexisting myofibrils. Our results taken together give new information on the morphological alterations which can be observed with both light and electron microscopic methods and will hopefully initiate further studies on the cause to DOMS. Furthermore the myofibrillar changes described in my thesis can for the first time explain the phenomena of the "second bout effects". Below I will discuss the main findings in more detail.

Changes in the sarcolemma integrity

Since plasma fibronectin staining was never observed within the muscle fibres, we conclude that the sarcolemma had not become permeable to fibronectin nor was there any appreciable disruption as a consequence of the three types of eccentric exercise leading to DOMS. Thus, the absence of fibronectin staining inside muscle fibres in the three different settings of eccentric exercise leading to DOMS reported here clearly indicates that sarcolemmal disruption of a magnitude that leads to muscle fibre degeneration and necrosis is not a feature of human skeletal muscles affected by muscle soreness. This does not mean that human muscle fibres can not undergo degeneration and necrosis if the insult is of an acute nature or is very intensive (e. g. after ultra-marathon race). In Crenshaw's study the necrosis was evident already one day after the race (Crenshaw et al., 1993). In the present study we did not observe any fibres containing macrophages at any time point. This is consistent with recent results described by Malm et al. (2000) who found that leukocytes do not migrate from blood to skeletal muscle in response to eccentric exercise and that exercise-induced skeletal muscle inflammation could not be confirmed (Malm, 2001).

Unexpectedly, we revealed an early influx of fibrinogen into muscle fibres of the biopsies taken 1 hour post exercise. The early influx of plasma protein into muscle fibres observed in the present study was suggested to reflect an early and transient form of sarcolemma injury induced by the exercise.

In animal models which were used to study the effects of eccentric contractions on muscles, sarcolemma injury has been revealed by staining for intracellular fibronectin (Lieber et al., 1994; Lieber et al., 1996; Friden and Lieber, 1998; Komulainen et al., 1998; Komulainen et al., 1999; Komulainen et al., 2000). Furthermore, some of these fibres showed discontinuous or loss of staining for dystrophin (Komulainen et al., 1998; Komulainen et al., 1999; Komulainen et al., 2000). The intracellular fibronectin and the disruption of dystrophin were seen early after eccentric contractions and prolonged for 7 days. We propose that the difference in the time course and extent of plasma protein infiltration between human and animals reflects the extent of damage occurred initially on the sarcolemma. Evidence has been shown that disruptions of cell membranes are common in vivo and that the membrane can reseal rapidly even in case of a disruption up to 1 μm in diameter (McNeil and Steinhardt, 1997). Normally membrane damage in living cells reseals within seconds to 1 min (Deleze, 1970; Steinhardt et al., 1994) and this rapid response is probably

required for survival. If the disruption of the sarcolemma is large, a segmental necrosis will be induced (Friden and Lieber, 1998).

Previous studies have shown that the cell wounding frequency observed in tissues in vivo and in vitro correlates with the level of mechanical stress which is imposed (McNeil & Steinhardt, 1997). This correlation is evident both in comparison of different tissues (muscle tissues have the highest measured levels compared with epithelia) and also in comparison with a single tissue differentially challenged with mechanical stress (see review McNeil & Steinhardt, 1997). The sarcolemma of skeletal muscle fibres plays a crucial part in force transmission. Force generated by myofibrils is transmitted across the sarcolemma to the extracellular matrix. Therefore, the sarcolemma is exposed to mechanical stress. Eccentric contractions have been demonstrated to generate high mechanical stress (Enoka, 1996), which has been proposed to cause muscle fibre membrane injury (Clarkson & Hubal, 2002; Lieber & Friden, 1999). In our study, despite the intracellular fibrinogen staining, none of the stainings for dystrophin or laminin was affected. We suggested that the sarcolemma get relatively minor defects which recovered rapidly, resulting only in the deposit of some fibrinogen in muscle fibres. As for the different staining patterns of the two plasma proteins, fibrinogen and fibronectin within individual fibers, it might reflect the different molecular mass of the two proteins [500 KD (fibronectin) vs 340 KD (fibrinogen)]. The sarcolemma injury might not allow fibronectin to enter into myofibres.

These data taken collectively indicate that partial loss of sarcolemma integrity is an early form of muscle damage induced by eccentric exercise and can be detected by identification of early plasma fibrinogen infiltration. As reversible injury to sarcolemma as shown here occurs in humans with DOMS whereas irreversible damage occurs in animal models thought to mimic human eccentric exercise-induced lesions (Armstrong et al., 1983; Lieber et al., 1994; Komulainen et al. 1998), we conclude that the animal models currently used are too harsh to be suitable to fulfill that purpose. Of special interest is whether this influx has some kind of stimulating – triggering effect on remodeling of the fibre and/or whether it causes edema as a part of the initiation of the swelling and palpation soreness.

Changes in the muscle cytoskeletal proteins and myofibrillar proteins

None of the post-exercise biopsies showed loss of staining for desmin. Instead, desmin longitudinal strands were observed in both the control and the post exercise biopsies. Using traditional immunohistochemical methods on human muscles with DOMS, Friden et al. (1984) observed similar longitudinal extensions of desmin between successive Z-discs in specimens taken 3 days after exercise, but not in those taken before exercise or 1 h post-exercise. The longitudinal strands were suggested to indicate either a de-generation or a re-generation of the myofibre induced by eccentric exercise. However, as shown in the present study, the desmin strands do not appear to be specifically linked to eccentric exercise, since they were observed to the same extent in the biopsies taken both before and after the exercise.

In the biopsies taken 2-3 days and 7-8 days post-exercise, the high-resolution immunohistochemistry revealed focal areas of increased staining for desmin and actin. The increased staining of the proteins reflects an increased synthesis or assembly the proteins, therefore suggests a myofibrilar re-generation or remodeling following the eccentric exercise.

Interestingly, in the areas where desmin and actin were increased, lacking staining for Z-disc related proteins, alpha-actinin, titin and nebulin, were observed. Theoretically the lack of staining observed here could either be caused by the degradation of these proteins in situ or could be the consequence of some kind of stress acting upon the myofibrillar Z-disc through yet unknown mechanisms which releases them from their "normal" binding sites on F-actin and other, more stable Z-disc components. In previous studies the

structural changes that occur in response to eccentric exercise have always been interpreted as muscle injury (Warren et al., 1999; Clarkson & Hubal, 2002; Lieber & Friden, 2002), probably supported by the pain experienced during muscle soreness that unconsciously is (and has been) interpreted as a sign for muscle damage (Miles & Clarkson, 1994; Clarkson & Newham, 1995). In this study, the observation of the co-localization of the increased staining for desmin and actin, and the lack of staining for alpha-actinin, titin and nebulin suggests that the two events might be correlated. The lack of the Z-disc-related proteins might be an initial event which triggers a subsequent synthesis or assembly desmin and actin.

In areas with supernumerary sarcomeres revealed by alpha-actinin, titin and nebulin, staining for desmin and F-actin was increasingly seen as multiple longitudinal strands. Instead of damage, these areas obviously reflect the insertion of new sarcomeres into pre-existing myofibrils. In previous studies, alterations of the Z-disc, like Z-disc streaming and smearing, have for a long time been thought to be the structural hallmark of muscle fibre damage induced by eccentric contractions (Friden et al., 1981; Newham et al., 1983; Nurenberg et al., 1992; Fielding et al., 1993; Gibala et al., 1995; Beaton et al., 2002; Feasson et al., 2002). In the earliest stages the Z-discs may show a wavy pattern, followed by an extension of dense material from the Z-disc further into the thin filaments, and finally the typical streaming throughout the sarcomeres with the concomitant loss of thick filaments (Carpenter & Karpati, 2001). Our immuno-fluorescence data, however, favors a different explanation. These so-called Z-disc streaming and smearing lack alpha-actinin, titin and nebulin, but instead they contain large amounts of actin and desmin. While in the centre mainly F-actin and desmin are revealed, which most likely corresponds to the longitudinal strands of electron-dense material identified earlier (Thornell et al., 1983), the peripheral regions contain alpha-actinin, titin and nebulin in various distributions ranging from a dotted pattern to true cross-striations. We conclude, therefore, that this staining pattern actually reflects stages of sarcomere formation.

Taken together, the findings of the different staining patterns observed in the present study have allowed us to propose a new model for myofibrillar remodeling upon eccentric exercise. The first step in this remodeling process seems to be the release of alpha-actinin, titin and nebulin (and most likely also other Z-disc- and I-band-associated proteins), whereas F-actin and desmin intermediate filaments persist. This local loss of certain actin-associated proteins might allow the recruitment of G-actin from cytoplasmic pools and the subsequent focal growth of actin filaments. The local loss of certain actin-associated proteins might also stimulate the de novo synthesis of desmin to form the observed longitudinal strands. The next step seems to be the re-integration of proteins like alpha-actinin and titin at regular distances to initiate an orderly transition from filament growth to the highly regular and more rigid sarcomeric organization of actin. Thus, actin and desmin filaments seem to form a principal mechanical and structural framework and proteins like alpha-actinin and titin participate in initiating sarcomeric periodicity. Nebulin, in contrast, appears to be integrated with a short delay.

In summary the present study provides us with a new insight into the process of adaptation to the increased strain that muscles experience during unaccustomed eccentric exercise. Thus, after a focal initial loss of some myofibrillar proteins, we can observe an addition of new sarcomeres into pre-existing sarcomeres. The processes involved show a recapitulation of the main aspects of de novo myofibril formation in the embryo. Eccentric exercise-induced myofibril alterations may therefore serve as a useful model for the study of adaptive processes involving sarcomerogenesis in situ in human skeletal muscle.

The hypothesis of sarcomerogenesis in muscles with DOMS is consistent with the findings of the “second bout effect”. A second bout of eccentric exercise, repeated within days to several months, results in

significantly less damage and soreness than the first bout (Clarkson & Tremblay, 1988; Ebbeling & Clarkson, 1989; Clarkson et al., 1992; McHugh et al., 1999; Stupka et al., 2001). The addition of new sarcomeres to the pre-existing myofibrils following the first bout of eccentric exercise make the myofibrils become more resistant to a second bout of similar eccentric exercise, thus showing less morphological changes.

Changes in the myofibril ultrastructure

Electron microscopy revealed four types of myofibrillar alterations: "amorphous widened Z-discs", "amorphous sarcomeres", "double Z-discs" and "supernumerary sarcomeres" in the biopsies taken 2-3 days and 7-8 days post-exercise. By comparing the myofibrillar ultrastructural alterations with the different staining patterns, we suggest that these ultrastructural alterations represent a process of myofibrillar remodeling rather than myofibrilar damage and confirm our light microscopic studies.

For the alterations of amorphous widened Z-discs of abnormally low electron-density, we interpret them as having lost some cross bridges and gained thin filaments. Similarly, the amorphous sarcomeres most likely correspond to the alterations of having an increase of F-actin filaments and lack of alpha-actinin, titin, nebulin and myosin (unpublished data). This is consistent with the immunohistochemical observations of focal loss of alpha-actinin, nebulin and titin staining in parallel with increased and widened staining for F-actin. In a recent study on human skeletal muscles, titin and nebulin were found to be reduced by 30% and 15%, respectively, 24 h after eccentric exercise (Trappe et al., 2002). The authors suggested that the structural components of the myofibrillar apparatus were degraded and the loss of these proteins may have important implications for the mechanisms regulating the adaptive response of skeletal muscle to resistance exercise.

In myology text books, Z-disk lesions of similar ultrastructural appearance as amorphous widened Z-discs are frequently referred to as early myofibrillar lesions (Engel, 1967; Morton, 1973; Carpenter & Karpati, 2001). They are seen in various myopathies and are often referred to as nonspecific degenerative abnormalities of the Z-disk (Engel & Banker, 1994; Carpenter & Karpati, 2001). In our study the amorphous widened Z-discs seem to reflect an initial alteration of the Z-disc due to the unusual strain imposed upon the muscle fibers during the eccentric exercise and in that sense might represent myofibrillar damage.

The double Z-discs are comparable to the F-actin broad bands with alpha-actinin, titin and nebulin located at the edges of the bands. The outer dense bands of the double Z-discs are interpreted to reflect that cross bridges composed of alpha-actinin are present. The density of the outer dense bands was identical to that of the Z-disc and matched the localization of alpha-actinin in broad F–actin bands. The appearance of nebulin and titin around the broad F-actin bands further supporting the hypothesis that the dense double bands represent two emerging Z-discs. By studying the effects of long-term eccentric exercise, Friden (1984) showed a figure of double Z-discs in which incorporation of nascent thick filaments into an individual myofibril were suggested. Our ultrastructural observation of a small M-band within the double Z-disc and the positive immunohistochemical staining for myomesin in the center of such sarcomeres (unpublished observations) are further evidence for an ongoing assembly of thick filaments into A-bands. Double Z-discs as a part of sarcomerogenesis have also been suggested in a study on heart Purkinje fibres (Thornell, 1973).

Supernumerary sarcomeres were observed both in electron microscopy and immunofluorescence staining. In the electron micrographs, longitudinal strands of filamentous material, extending from one Z-disc through one or more sarcomeres and ending at Z-disc level were observed in such areas. Notably the density of these longitudinal strands was in general lower than that of the Z-discs. Previous studies have shown that the electron-density of the Z-discs are mainly related to alpha-actinin,

which in human muscle forms two or more repeats of cross bridges connecting the actin filaments from opposite sarcomeres (Luther & Squire, 2002). Recent studies have shown that a number of other proteins are present in the Z-discs and they might also contribute to the density (Clark et al., 2002). Two of these are titin and nebulin. In light microscopy the typical features of supernumerary sarcomeres were the increased staining for F-actin and desmin as longitudinal strands. In general these strands originated from a Z-disc and extended longitudinally for one or more sarcomeres and ended at the Z-disc level. The stainings of alpha-actinin, titin and nebulin always revealed a sarcomeric Z-disk pattern and detectable levels of these proteins were never co-localized with desmin or F-actin in the longitudinal strands. Our conclusion is that alpha-actinin, titin and nebulin do not contribute to the electron-dense longitudinal strands but that desmin and F-actin do. The terms Z-disc streaming and Z-disc smearing, which both have been used to describe this kind of alterations, are misnomers since the alterations do not reflect an expansion of the Z-disc itself. Our immunohistochemical results showed that the sarcomeric proteins alpha-actinin, nebulin and titin are localized in supernumerary sarcomeres, which are similar to those of developing myofibrils. This strongly favors the hypothesis that supernumerary sarcomeres represent sarcomerogenesis (Schmalbruch, 1968; Thornell et al., 1983).

Muscle fibre ultrastructural changes induced by eccentric contractions have been reported in many studies. However, careful examinations of the pictures which showed the typical Z-disc streaming in these studies revealed that many of them contained supernumerary sarcomeres (Newham et al., 1983; Friden et al., 1984; Nurenberg et al., 1992; Roth et al., 2000). Focal and extensive Z-disc streaming have also been observed in biopsies of healthy young people (Meltzer et al., 1976), which (Figs. 3 and 4) undoubtedly also show areas with supernumerary sarcomeres.

In conclusion, ultrastructural alterations do occur after unaccustomed eccentric exercise. However by taking into account of the protein composition of the alterations observed by immunohistochemistry, we propose that they reflect myofibril remodeling rather than myofibril damage. The amorphous widened Z-discs and the amorphous sarcomeres can be related to an increased expression of F-actin and a disruption of the Z-disc in the sense that alpha-actinin, titin, nebulin and possibly other Z-disc proteins are detached or degraded from the Z-disc. Furthermore the Z-disc streaming and smearing are not caused by a proliferation of the Z-disc-related proteins; instead they are due to an increase of F-actin and desmin. Double Z-discs and areas with supernumerary sarcomeres do not reflect myofibril damage but can be related to sarcomerogenesis and remodeling of the myofibrils.

CONCLUSIONS

The present study has revaluated the morphological ultrastructural changes observed in biopsies from persons with exercised-induced muscle soreness using immunohistochemical methods. The main conclusions are:

- There is an early and temporally injury of the sarcolemma but no muscle fibre necrosis followed by inflammation in human muscles with DOMS.
- The intermediate filament cytoskeleton is not disrupted and desmin is not degraded in human muscles with DOMS. On the contrary the focal increase of staining for desmin must be due to an increased synthesis of the protein. The longitudinal desmin strands connecting Z disc in longitudinal register are proposed to stabilize sarcomeres under reconstruction.
- The myofibrillar alterations seen in post-exercise biopsies are not a myofibrillar disruption but a remodeling of myofibrils by the addition of new sarcomeres within the myofibrils. This leads to a lengthening of the myofibrils, which for the first time gives a mechanistic explanation to the "second bout effect".
- The Z-disk is the myofibrillar locus for the initial changes in the myofibrils, most likely due to the high strain induced by the repetitive eccentric contractions. Polymerization of G-actin to F-actin seems to be one major initial alteration in DOMS-affected muscles. Temporally loss of alpha-actinin, titin and nebulin are other changes. Which event comes first can so far only be speculated on. Before a sequential order can be proposed, further analyses are needed to reveal which additional proteins and signaling pathways are involved.
- The proposal of Armstrong et al. (Armstrong et al., 1983) in 1983 "while there is no proof that morphological damage in rodent muscle and delayed muscle soreness in human subjects are manifestation of the same exercise-induced cellular events, it is reasonable to suspect they are" is shown to be invalid. The current hypotheses on the pathogenesis of DOMS based on animal models have to be discarded as they do not mimic DOMS in humans.

The present study will change the dogma that myofibrillar disruption/damage is a hallmark of DOMS. This finding is of clinical importance as the myofibrils contrary to becoming weakened, are reinforced by cytoskeletal elements during the addition of new sarcomeres. The latter gives for the first time a mechanistic explanation for the lack of further damage upon additional exercise (second bout effect). Furthermore, the current methods of analysis of biopsies from eccentric exercised subjects can be used as an in situ model to analyze the molecular changes taking place in the muscle fibres affected by DOMS.

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