Mechanical loading regulates the expression of tenascin-C in the myotendinous junction and tendon but does not induce de novo synthesis in the skeletal muscle

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Summary

Tenascin-C is a hexabrachion-shaped matricellular protein with a very restricted expression in normal musculoskeletal tissues, but it is expressed abundantly during regenerative processes of these tissues and embryogenesis. To examine the importance of mechanical stress for the regulation of tenascin-C expression in the muscle-tendon unit, the effects of various states of mechanical loading (inactivity by cast-immobilization and three-varying intensities of subsequent re-activity by treadmill running) on the expression of tenascin-C were studied using immunohistochemistry and mRNA in situ hybridization at the different locations of the muscle-tendon unit of the rat gastrocnemius muscle, the Achilles tendon complex. This muscle-tendon unit was selected as the study site, because the contracting activity of the gastrocnemius-soleus muscle complex, and thus the mechanical loading-induced stimulation, is easy to block by cast immobilization.

Tenascin-C was expressed abundantly in the normal myotendinous and myofascial junctions, as well as around the cells and the collagen fibers of the Achilles tendon. Tenascin-C expression was not found in the normal skeletal muscle, although it was found in blood vessels within the muscle tissue. Following the removal of the mechanical loading-induced stimulation on the muscle-tendon unit by cast immobilization for 3 weeks, the immonoreactivity of tenascin-C substantially decreased or was completely absent in the regions expressing tenascin-C normally. Restitution of the mechanical loading by removing the cast and allowing free cage activity for 8 weeks resulted in an increase in tenascin-C expression, but it could not restore the expression of tenascin-C to the normal level (in healthy contralateral leg). In response to the application of a more strenuous mechanical loading stimulus after the removal of the cast (after 8 weeks of low- and high-intensity treadmill running), the expression of tenascin-C was markedly increased and reached the level seen in the healthy contralateral limb. Tenascin-C was abundantly expressed in myotendinous and myofascial junctions and in the Achilles tendon, but even the most strenuous mechanical loading (high-intensity treadmill running) could not induce the expression of tenascin-C in the skeletal muscle. This was in spite of the marked immobilization-induced atrophy of the previously immobilized skeletal muscle, which had been subjected to intensive stress during remobilization. mRNA in situ hybridization analysis confirmed the immunohistochemical results for the expression of tenascin-C in the study groups.

In summary, this study shows that mechanical loading regulates the expression of tenascin-C in an apparently dose-dependent fashion at sites of the muscle-tendon unit normally expressing tenascin-C but can not induce de novo synthesis of tenascin-C in the skeletal muscle without accompanying injury to the tissue. Our results suggest that tenascin-C provides elasticity in mesenchymal tissues subjected to heavy tensile loading.

Key words: Tenascin-C, Mechanical strain, Fibronectin, Skeletal muscle, Tendon, Cartilage, Bone, Tensile, Elastic, Extracellular matrix, Adhesion, Integrin

Introduction

The composition of extracellular matrix (ECM) of the musculoskeletal tissues appears to be controlled by the mechanical stresses placed on the cells within the connective tissue (Banes et al., 2001). Tenascin-C (TN-C) is a six-armed hexabrachion-shaped ECM glycoprotein initially discovered at the myotendinous junction (MTJ) (Erickson, 1993; Erickson, 1997; Chiquet-Ehrismann, 1995; Mackie and Tucker, 1999; Jones and Jones, 2000a; Jones and Jones, 2000b; Järvinen et al., 2000). It is a member of a family of related proteins

comprising TN-C, tenascin-R, tenascin-X, tenascin-W and tenascin-Y (Jones and Jones, 2000a). Tenascins, in turn, belong to a specialized class of ECM proteins called the matricellular proteins (Murphy-Ullrich, 2001; Sage, 2001). Whereas most of the ECM glycoproteins promote cell adhesion and cause cytoskeletal reorganization, matricellular proteins (tenascins, thrombospondins and SPARC) function as adaptors and modulators of cell-matrix interactions (Murphy-Ullrich, 2001; Sage, 2001). The key feature of the matricellular proteins is that they act as both soluble and insoluble proteins (Murphy-Ullrich, 2001; Sage, 2001). Like the matricellular proteins (Murphy-Ullrich, 2001; Sage, 2001), the function of the TN-C seems complex, as it takes part in such opposing phenomena as cell adhesion and migration (Erickson, 1993; Erickson, 1997; Jones and Jones, 2000a; Jones and Jones, 2000b). To further complicate the issue, a recent extensive characterization of the structure of the TN-C protein has provided new insights into its adhesive and migrational characteristics: a single molecule of TN-C possesses elastic properties and is capable of stretching to several times its resting length (Oberhauser et al., 1998). In addition, the epidermal growth factor (EGF)-like repeats of TN-C can act as ligands for the EGF receptor and subsequently can activate growth factor receptors (Swindle et al., 2001).

TN-C is abundantly expressed in the musculoskeletal tissues during organogenesis and embryogenesis (Kardon, 1998; Jones and Jones, 2000a; Jones and Jones, 2000b) but somewhat less abundantly in the mature forms of these tissues (Kannus et al., 1998a). However, mature musculoskeletal tissues seem to be unique in their expression pattern of TN-C: TN-C is expressed abundantly in all musculoskeletal regions transmitting high mechanical forces from one tissue component to another, for example, in myotendinous and osteotendinous junctions (Swasdison and Mayne, 1989; Hurme and Kalimo, 1992; Salter, 1993; Chevalier et al., 1994; Mackie and Ramsey, 1996; Riley et al., 1996; Webb et al., 1997; Kannus et al., 1998a; Mackie et al., 1998; Järvinen et al., 1999; Järvinen et al., 2000; Ireland et al., 2001; Theilig et al., 2001; Altman et al., 2002; Martin et al., 2003; Hadjiargyrou et al., 2002). Not surprisingly, it was shown that TN-C expression is elevated in fibroblasts cultured in stressed collagen gels (Chiquet-Ehrismann et al., 1994; Chiquet, 1999; Kessler et al., 2001). And at least two different transcription factors and TN-C promoter elements responding to mechanical loading have been identified; a conserved GAGACC stretch-responsive enhancer region (Chiquet, 1999) and a separate signal transduction pathway that involves matrix metalloproteinases (MMPs) and Egr-1 transcription factor (Jones et al., 2002).

We recently extended these in vitro findings by showing that mechanical stress also regulates the expression of TN-C in vivo (Järvinen et al., 1999). In addition, other groups showed that mechanical loading placed upon the tissues controls TN-C expression in the periosteum, heart, blood vessels, skin wounds, ligament, osteotendinous junction, during synovial joint formation and in skeletal muscle (Webb et al., 1997; Costa et al., 1999; Feng et al., 1999; Yamamoto et al., 1999; Flück et al., 2000; Mehr et al., 2000; Mikic et al., 2000; Theilig et al., 2001; Jones et al., 2002; Satta et al., 2002; Altman et al., 2002; Martin et al., 2003).

In this study we investigated whether TN-C expression is influenced by different mechanical loading states in a normal muscle-tendon unit. To study this, we first removed the mechanical loading-induced stimulus from the gastronemius-Achilles tendon complex of a rat by immobilizing its hindlimb in a cast for 3 weeks, and then restored the stimulus by subjecting rats to three exercise protocols differing in their intensity (free cage activity, low- and high-intensity treadmill running). The muscle-tendon unit of the rat calf muscles is especially suited for studying the regulation of TN-C expression, as it contains both regions expressing TN-C abundantly (the MTJ between the muscle and the Achilles tendon and the Achilles tendon) and regions devoid of TN-C (e.g. the skeletal muscle, where TN-C is only expressed around the blood vessels). The mechanical strains generated by the contracting gastrocnemius muscle are first concentrated on the MTJ (a specialized structure tailored for transmitting the mechanical forces generated by muscle contractions into more rigid tendon tissue) and then on the Achilles tendon itself (the strongest tendon in the rat body).

A special emphasis was placed on a recent, somewhat surprising, finding by Flück et al. suggesting that strenuous mechanical loading induces de novo synthesis of TN-C in the skeletal muscle itself (Flück et al., 2000). To specifically explore this hypothesis, the rat gastrocnemius-muscle–Achilles-tendon complex was first subjected to 3 weeks of inactivity (cast immobilization) to induce a severe atrophy of the tissues and, thus, to ascertain that the subsequent restitution of mechanical loading would subject the tissues to sufficient stress to confirm (or oppose) the hypothesized de novo synthesis of TN-C in the skeletal muscle.

Materials and Methods

Study groups and experimental protocol

60 adult male rats of the Spraque-Dawley strain (University of Tampere, Tampere, Finland) were used in the study. At the beginning of the study, the rats were between 9 and 11 weeks old with a body weight of 355±26 g. They were fed with standard laboratory pellets and water ad libitum. The ‘Guiding Principles in the Care and Use of Animals’ of the American Physiological Society were followed and the study design, including descriptions of the procedures used for anesthesia and killing of the rats, was approved by the ethics committee of the University of Tampere.

The rats were divided into six groups (Fig. 1), of which two were control groups (C_3 and C_{11}) and the other four were the study groups (IM_3, FR_{11}, LR_{11} and HR_{11}). In the control groups, the animals were allowed to move freely in the cage (18×35×55 cm), five animals per cage, and the gastrocnemius muscles and the Achilles tendons were analyzed 3 weeks (C_3) and 11 weeks (C_{11}) after the starting point (Fig. 1). The rats were killed using carbon dioxide inhalation, and the calf muscle complex (including the gastrocnemius and soleus muscles and the Achilles tendon) was removed from both limbs immediately after death.

At entry, the left hind limb of each study animal (groups IM_3, FR_{11}, LR_{11} and HR_{11}) was immobilized with a padded tape from toes to above the knee. The knee was fixed in 100° flexion and the ankle in 60° plantarflexion. The fixation was checked daily. The immobilization method has been described in detail elsewhere (Józsa et al., 1990). The right hind limb was kept free, and its gastrocnemius–Achilles-tendon complex served as an internal control.

After 3 weeks, the rats of the group IM_3 (the immobilization group) were sacrificed, and the samples were taken as described above (Fig. 1). In the remaining groups FR_{11}, LR_{11} and HR_{11} (the remobilization groups), the tape was removed and the animals were allowed to remobilize the left hind limb for 8 weeks. The group FR_{11} rats (the

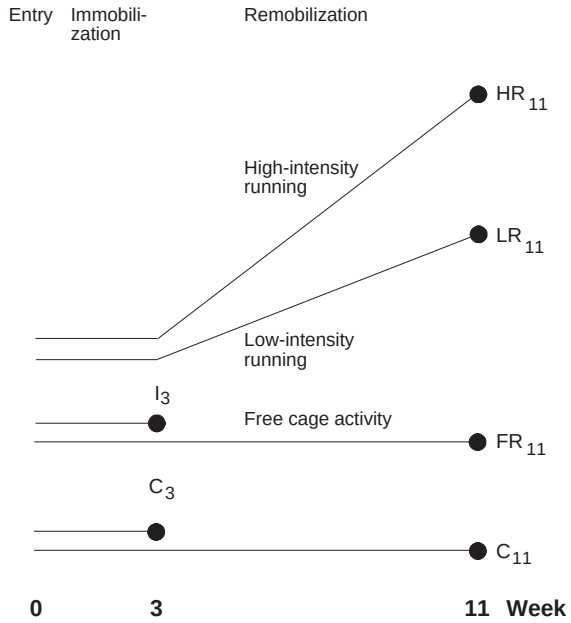

Fig. 1. The experimental design of the study. C, control group at 3 weeks; IM, immobilization group at 3 weeks; C, control group at 11 weeks; FR, free remobilization group at 11 weeks; LR, low-intensity running group at 11 weeks; HR, high-intensity running group at 11 weeks.

free remobilization group) moved freely in the cage and no additional physical training was used.

Rats in the groups LR_{11} and HR_{11} were allowed to move freely in their cage for 1 week, after which they started to run on a treadmill twice a day, 5 days a week for 7 weeks. In the group LR_{11} (the group with a low intensity running program), the speed of the treadmill was 20 cm/second with an inclination of 10°. During the first running week, there was only one 20 minute session per day, after which there were two sessions per day (the morning and afternoon sessions at least 5 hours apart) for 6 weeks. The program was progressive so that the running time increased from 20 minutes per session in the first 2 weeks to 45 minutes per session in the last week.

In the group HR_{11} (the group with a high intensity running program), the speed of the treadmill was 30 cm/second with an uphill inclination of 30°. This final speed and inclination was achieved by a gradual increase in speed and inclination during the first week of running. As in the group FR_{11}, there was only one daily session during the first running week, and two thereafter. The running was also progressive, from 20 minutes per session in the first two weeks to 45 minutes per session in the 7^{th} and 8^{th} weeks.

After the remobilization period of 8 weeks (Fig. 1), the remobilized animals in the groups FR_{11}, LR_{11}, and HR_{11} were also sacrificed using carbon dioxide and the calf muscle–Achilles-tendon complexes were dissected.

Sample preparation

The dissected gastrocnemius-muscle–Achilles tendon complex (including the calcanear insertion and thus the osteotendinous junction) were cleared of the adherent fat and connective tissue and transversely divided in half. The proximal half of the muscles were snap-froxen immediately in freon 22 cooled with liquid nitrogen and stored at –35°C until processing and analysis, whereas the distal half (fixed at the resting length by attaching the samples by pins to pieces of cork) was fixed in neutral buffered 6% formalin (pH 7.4) and embedded in paraffin. Both ends of the muscles were attached with pins to the underlying piece of cork to ensure that the normal resting length of the muscle was maintained throughout the fixation and embedding process.

TN-C expression in the gastrocnemius-muscle–tendon unit

Immunohistochemistry

10 to 15 serial longitudinal 5 μm thick sections were cut from the middle area of each formalin-fixed paraffin-embedded block, the cutting surface being sagittal (not frontal). Half of the serial longitudinal sections were stained with hematoxylin-eosin or with modified Herovici method, and the other half were used for the immunohistochemical examination.

For immunohistochemistry, polyclonal rabbit anti-human tenascin-C (dilution 1:1600) (Telios Pharmaceuticals, Inc., San Diego, CA) was used as a primary antibody (Hurme and Kalimo, 1992; Kannus et al., 1998a; Järvinen et al., 1999). TN-C antiserum is crossreactive with the corresponding rat antigen (manufacturer's information), and we have previously verified its specificity for TN-C (Järvinen et al., 1999).

The bound primary antibody was visualized using the appropriate avidin-biotin-peroxidase method (Vectastain, Vector Laboratories, Burlingame, CA or Histostain Plus-kit, Zymed Laboratories, San Francisco, CA) with diaminobenzidine as a chromogen. After the immunohistochemical reaction, sections were counterstained with hematoxylin. Negative control sections (i.e. specimens incubated with rabbit serum or without the polyclonal antibody) were included in every staining patch. Normal rat skeletal muscle with intact myotendinous junctions, as well as its injured counterpart, expressing TN were used as positive controls (Hurme and Kalimo, 1992; Kannus et al., 1998a). Finally, all the histological sections were analyzed and photographed with a light microscope.

mRNA in situ hybridization

In situ hybridization analysis was performed on several samples from each study group. Paraffin sections (10 μm) were cut onto Superfrost Plus (Menzel, Germany) slides. Two synthetic oligonucleotide probes directed against TN-C mRNA [nucleotides 609-642 and 1531-1564, GenBank accession U09361 (LaFleur et al., 1994)] were labeled with a specific activity of 1×10^9 cpm/ug at the 3′ end with ^{33}P-dATP (DuPont-New England Nuclear Research Products, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Int., Buckinghamshire, UK). After the xylene and graded alcohol series, the sections were washed in water, air dried and hybridized at 42°C for 18 hours with 5 ng/ml of the probe in the hybridization cocktail, washed four times (15 minutes each) in 1×SSC at 55°C, and while in the final rinse, left to cool to room temperature (for an approximately 1 hour) (Järvinen et al., 1996; Kononen and Pelto-Huikko, 1997). Autoradiograph films (Amersham β-max; Amersham Int., Buckingshire, UK) were overlaid on slides, exposed for three weeks and then developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY). Histology was controlled afterwards by staining the hybridized tissue sections with hematoxylin.

Gross characteristics of the gastrocnemius muscle-tendon unit

To provide a broader context for the possible changes in the expression of TN-C, a comprehensive series of microscopic analyses characterizing the changes induced by altered mechanical loading on the calf-muscle–Achilles tendon complex of the rats was performed.

Histochemistry, histology and immunohistochemistry

Capillary density and cross-sectional area of muscle fibers

Unfixed serial cryostat cross-sections (6 μm in thickness) were obtained from frozen muscles and stained for myofibrillar ATPase

activity, after preincubation at pH 4.2, 4.6 and 10.2 (Józsa et al., 1993; Kannus et al., 1998b). This staining procedure allowed the identification of muscle fibres, as type I, type IIA (fast-twitch oxidative glycolytic) or type IIB (fast-twitch glycolytic), the measurement of fiber cross-sectional area and the identification of the intramuscular capillaries (Józsa et al., 1993). In each muscle, 300-500 consecutive neighboring capillaries and the number of simultaneously occurring muscle fibers were calculated from the above-described ATPase-stained sections (pH 4.2 and 4.6). The cross-sectional area was determined both for type I and type II fibers as previously described (Kannus et al., 1998b; Kannus et al., 1998c). The oxidative enzyme activity of the fibres was demonstrated by the NADH reductase reaction (Kannus et al., 1998b; Kannus et al., 1998a). The remaining cryostat sections were stained with periodic-acid–Schiff (PAS), with and without diastase pretreatment, to detect the glycogen content of the muscle fibres.

From the paraffin blocks, 5 μm thick serial sections were cut and stained with hematoxylin-eosin, picrosirius and phosphotungstic-acid–hematoxylin for the evaluation of the intramuscular connective tissue and pathological fiber alterations as described elsewhere (Kannus et al., 1998b). Picrosirius stained the connective tissue (endo-, peri- and epimysium) dark red, which contrasts well with the pale yellow muscle fibres. From each muscle, two to three picrosirius-stained cross-sections were examined using a Zeiss microscope and were analyzed using a system consisting of a video camera, automatic image analyzer and image software (Muscle Image Analysis System, IBM-KFKI, Budabest, Hungary) (Kannus et al., 1998b). In each section, the connective tissue and muscle fiber areas were recorded by measuring the optical density of 442,400 points in a microscopic field (~0.86 mm in ×160 magnification). The percentage of connective tissue or connective tissue to muscle fiber was calculated from the ratio of total connective tissue area to muscle fiber area and expressed as a percentage. To calculate the mean connective tissue area for each muscle, 10-30 images/muscle were analyzed (two to three sections/muscle including 2-10 fields/section). Fields containing blood vessels other than capillaries were excluded from the analysis.

Pathological fiber alterations

The number (and percentage, %) of fibers with a pathgological, morphological and histochemical alteration was determined by analyzing 500 consecutive neighboring type II fibers from each control and from experimental gastrocnemius muscle (Kannus et al., 1998b). The above-described NADH reductase, PAS, ATPase and phosphotungstic-acid–hematoxylin preparates were used for these analyses.

According to their characteristic histological and histochemical features, the alterations were classified as follows (Kannus et al., 1998b): a moth-eaten fiber (referring to spiral-type deformation and destruction of the myofibrillar network of the fiber, the term being derived from the microscopic moth-eaten appearance of the fiber); a central core formation within the fiber (referring to abnormally increased oxidative enzyme activity and abnormal aggregation of the myofibrils in the central area of the fiber); a loss of oxidative enzyme activity in the central part of the fiber (referring to a reduced number of mitochondria and thus reduced aerobic energy production in that area of the fiber); an increased oxidative enzyme activity in the peripheral areas of the fiber (referring to the increased number of mitochondria and thus increased aerobic production in that area of the fiber); a shell-like fiber (referring to shell-like degradation and degeneration of the myofibrillar network of the fiber, the term being derived from the microscopic shell-like appearance of the fiber); a fiber splitting; any other (undetermined) alteration; and multiple alterations. The total percentage of fibers with a pathological alteration was also calculated for each control and experimental muscle.

Visualization and histometric quantification

To eliminate any bias on the part of the observer during the analyses described, all data collection and all examinations were performed on a blind basis with respect to treatment group assignment.

Statistical analysis

For the continuous outcome variables, statistical comparisons were first done using a two-way ANOVA, with the rat group and hindlimb side being the grouping variables. When the two-way ANOVA indicated significant (P<0.05) group and side differences and significant (P<0.05) group-side interactions, Tukey's post hoc analyses were used for pairwise comparisons. In the frequency outcome variable (the percentage of pathological fiber alterations), the groups were compared with the χ^2 test. The given significance levels refer to two-tailed tests. The sample size (8 rats/group with both of the hindlimbs analyzed) required to detect a 10% difference in muscle morphology between the experimental and control groups was based on a power analysis by using alpha=0.05 and beta=0.20 (power 0.80).

Results

Gross characteristics of the gastrocnemius muscle-tendon unit

Normal histology and the effects of inactivity and subsequent restitution of mechanical loading on the rat calf-muscle–Achilles tendon complex (Groups C_3 and C_{11})

The effects of immobilization, free mobilization and low- and high-intensity treadmill running on the percentage area of intramuscular connective tissue, capillary density, muscle fiber cross-sectional area and pathological muscle fiber alterations are presented in Fig. 2A-D and Table 1. In all of these conditions, the two-way ANOVA indicated significant group and side differences in the gastrocnemius muscle (P<0.001 for both differences), as well as a significant group-side interaction (P<0.001 for gastrocnemius muscle), and, therefore, Tukey's post hoc analyses were also performed for all parameters (see below).

The measured variables were selected on the basis of their established role as markers for physical-inactivity-induced

Table 1. Percentage of type II muscle fibers with a pathological alteration of all fibers examined in immobilized or immobilized-remobilized gastrocnemius muscles

Fiber alteration	C	IM_3	FR_{11}	LR_{11}	HR_{11}	P value*
Moth-eaten fiber	0	6	7	1	0	<0.01
Central core formation	0	4	5	1	0	<0.05
Loss of oxidative enzyme activity in central part of fiber	<1	7	4	2	0	<0.05
Increased oxidative enzyme activity in peripheral areas of fiber	1	8	6	2	2	<0.05
Shell-like fiber	0	4	2	1	0	NS
Fiber splitting	1	2	4	4	6	NS
Other alteration	<1	2	3	1	0	NS
Multiple alterations	<1	4	5	1	2	NS
Total number of abnormal fibers,%	4	37	36	13	10	<0.001

Values are percentages. * χ^2 test.

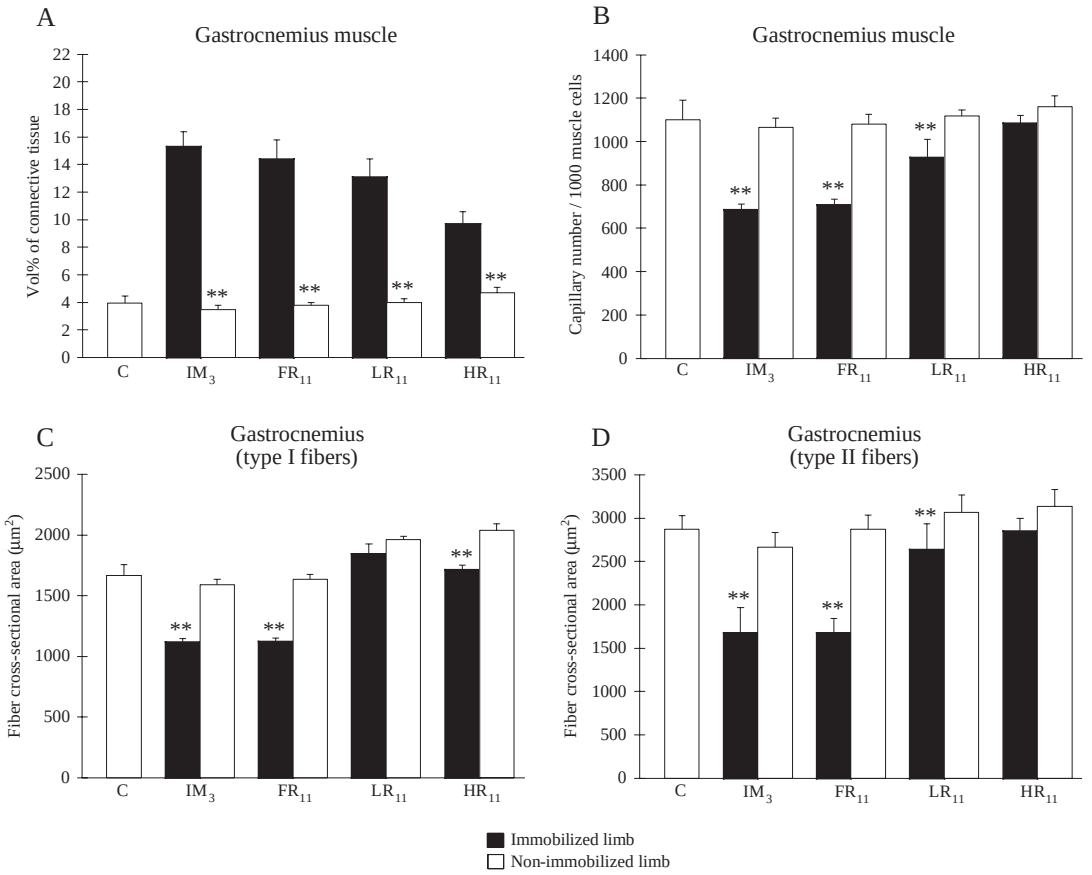

Fig. 2. (A) The percentage area of intramuscular connective tissue, (B) capillary density and (C) cross-sectional area of type I and (D) type II fibers of the rat gastrocnemius muscle after inactivity and restitution of mechanical loading (three different intensities). Values are means±s.d. Group abbreviations are as defined in Fig. 1. Significant immobilized-to-non-immobilized hindlimb differences, $^{**}P<0.05$. For other comparisons, see text.

atrophy of skeletal muscle (Appell, 1990; Józsa et al., 1990; Kannus et al., 1992; Kannus et al., 1998b; Kannus et al., 1998c; Järvinen et al., 2002); that is, capillary density as well as the muscle fiber cross-sectional area go through a substantial decrease in response to physical inactivity, whereas the simultaneous increase in the amount of intramuscular connective tissue takes place in atrophic skeletal muscle (Appell, 1990; Józsa et al., 1990; Kannus et al., 1992; Kannus et al., 1998b; Kannus et al., 1998c; Järvinen et al., 2002). The measured fiber alterations, in turn, are morphological changes that have been reported to take place in atrophic skeletal muscle fibers (Appell, 1990; Józsa et al., 1990; Kannus et al., 1992; Kannus et al., 1998b; Kannus et al., 1998c).

Percentage area of connective tissue

In the gastrocnemius muscle of the contralateral control (non-immobilized) hindlimbs, the group differences in the amount of connective tissue were small and insignificant, the percent area being 4.0% in the control group, 3.5% in the IM_3-group, 3.8% in the FR_{11}-group, 4.0% in the LR_{11}-group, and 4.7% in the HR_{11}-group, respectively (Fig. 2A). By contrast, immobilization of the left hindlimb for three weeks created a large and significant ($P<0.01$) side-to-side difference in the mean area of the connective tissue, the area of the connective tissue being 15.3% in the immobilized left hindlimb (Fig. 2A). Free cage activity (FR_{11}) and especially low- and high-intensity treadmill running for eight weeks significantly ($P<0.01$) restored this value to control levels (14.4%, 13.1% and 9.7%, respectively), the restorative effect being significantly better in the running groups ($P<0.01$ for both LR_{11}- and HR_{11}-groups) than in the free cage activity group (FR_{11}) (Fig. 2A). Despite the strenuous nature of the remobilization protocols, the side-to-side difference was still significant in all three remobilization groups ($P<0.001$ for all groups) as the amount of the connective tissue was 3.8-, 3.3- and 2.1-fold in the previously immobilized muscles in comparison to the healthy contralateral muscles (Fig. 2A).

Capillary density

In the gastrocnemius muscle of the healthy, contralateral hindlimbs, the group differences in the capillary density were small and nonsignificant, although the animals that had gone through the intensive remobilization protocols tended to have (not statistically significant) a higher capillary density than that in the rats with sedentary lifestyle (free cage activity or immobilization) (Fig. 2B). Immobilization produced a significant ($P<0.001$) side-to-side difference in the gastrocnemius muscle capillary density, the density of the immobilized left hindlimb being only 64% of the healthy contralateral gastracnemius muscle (and 62% of the control, C_3) (Fig. 2B). Free cage activity (FR_{11}) of 8 weeks did not improve the situation at all (left, immobilized hindlimb density was still only 66% of that of the contralateral hindlimb ($P<0.001$ and 64% of the control, C_{11}), but in the LR_{11} group, the capillary density of the previously immobilized left gastrocnemius muscles had improved substantially and had

even reached the control level in the HR_{11} group (Fig. 9B). In the treadmill-trained animals, the side-to-side difference of 17% was still significant ($P<0.01$) in the LR_{11}-group and remained 6% lower in the HR_{11}-group ($P<0.05$) (Fig. 2B).

Fiber cross-sectional area

In the gastrocnemius muscles of the non-immobilized hindlimbs, the mean cross-sectional area of type I fibers was, as expected, significantly higher in the LR_{11}- and HR_{11}-groups ($P<0.01$ for both groups) than in the other groups (Fig. 2C). The immobilization period resulted in a significant side-to-side difference, the mean left (immobilized) hindlimb cross-sectional area being only 70% of the contralateral (non-immobilized) in the gastrocnemius muscle ($P<0.001$) (Fig. 2C). Free cage activity did not improve the situation (the left-side area having only 69% of the cross-sectional area of the right hindlimb) ($P<0.001$), whereas after the treadmill training, the hindlimb cross-sectional areas had reached the age-matched control levels, being 111% (LR_{11}) and 103% (HR_{11}) of that in the controls (both NS) (Fig. 2C). However, a left-to-right difference still existed in the gastrocnemius muscles of the HR_{11}-group ($P<0.01$) because of the positive effect of running on the cross-sectional area (of type I fibers) in the non-immobilized (right) hindlimbs (Fig. 2C).

Immobilization also produced a significant side-to-side difference in the cross-sectional area of the type II fibers, the cross-sectional area of the immobilized hindlimb being 63% of that in the right hindlimb ($P<0.01$) (Fig. 2D). Free cage activity could not improve the situation, the left hindlimb value being 58% of that in the right hindlimb ($P<0.01$). However, the cross-sectional area of type II muscle fibers of the previously immobilized hindlimb muscles reached the control level after low- and high-intensity treadmill running (92% in the LR_{11}- and 99% in the HR_{11}-group as compared to C_{11}), but, similar to type I fibers, a clear side-to-side difference still existed in both of these groups because of the positive effect of running on the non-immobilized (right) hindlimb muscles ($P<0.01$) (Fig. 2D).

Pathological fiber alterations

The total number of fibers with a pathological alteration was very low in the gastrocnemius muscle in the control rats (4%) (Table 1). Immobilization resulted in a significant increase in the amount of pathological fibers (37%, $P<0.001$), and the situation did not improve in the free cage activity animals (36%). In the LR_{11}- and HR_{11}-groups, the percentage of the fibers with a pathological alteration was clearly lower than that in the IM_3- and FR_{11}-groups (13% in the LR_{11}- and 10% in the HR_{11}-group, respectively), but the above-described control level was not completely attained even in the HR_{11}-group (Table 1).

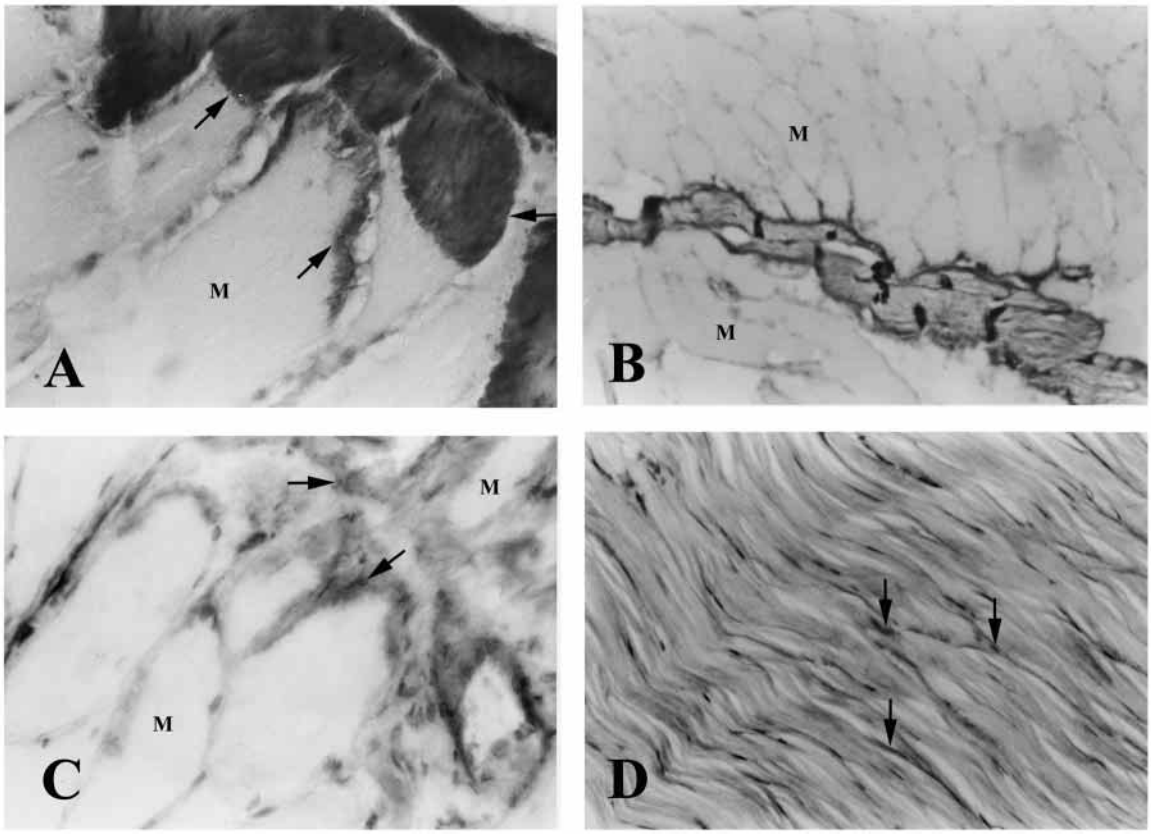

Fig. 3. (A) The expression of TN-C can be seen both at the MTJ and the microtendon (arrows). (B) Strong expression of TN-C can also be detected in the myofascial junction, whereas the endomysial and perimysial connective tissues are negative for TN-C immunoreactivity. (C) Only TN-C immunoreactivity that can be detected in the skeletal muscle is visible in the intramuscular myofiber-myofiber junctions at the tips of muscle cells (arrows). (D) In the tendon, immunohistochemical expression of TN-C is visible in the tenocyte–collagen-fiber interface (arrows) as well as on the surface of the collagen fiber bundles of the Achilles tendon. Magnification: A,C,D ×100, B ×50.

TN-C expression in the gastrocnemius muscle-tendon unit

Normal expression of TN-C in the gastrocnemius muscle, MTJ and Achilles tendon (Groups C_3 and C_{11})

In the gastrocnemius muscle of the control rats, both the myofibers and the endo- and perimysium stained negative for TN-C (Fig. 3A,B). The only TN-C immunoreactivity seen in the gastrocnemius muscle was a few positive stainings of the myofiber-myofiber (myomuscular) junctions (Fig. 3C). In addition to this TN-C immunoreactivity, TN-C was also identified in the intramuscular blood vessels. However, a strong, irregular band of TN-C immunoreactivity was found at the interface of the MTJ, and, additionally, the microtendon part of the MTJ also stained strongly for TN-C (Fig. 3A). A band of TN-C immunoreactivity was also seen in the myofascial junction (Fig. 3B), whereas no TN-C was identified in the microtendon roots extending from the MTJ to the lateral sides of the muscle fibers. TN-C could also be visualized by mRNA in situ hybridization in the MTJ and myofascial junction (MFJ).

In the normal Achilles tendons, TN-C was detected in small amounts in the tenocyte collagen fiber interface (Fig. 3D) and on the surface of the parallel-oriented collagen bundles of the tendon (Fig. 3D). Intense TN-C immunoreactivity was seen in the tendon, fibrocartilage and mineralized fibracartilage zones of the osteotendinous junction (OTJ) of the Achilles tendon, but none was seen in the actual bone tissue. mRNA in situ hybridization analysis confirmed the strong expression of TN-C in the tendon (Fig. 4C).

Inactivity-induced changes in the expression of the TN-C (Group IM_3)

After three weeks of immobilization, the muscle cells and their sarcolemmal membrane stained negative for TN-C in the immobilized and contralateral (non-immobilized) gastrocnemius muscles (Fig. 5A). The MTJs of the immobilized muscles expressed no or only very mild TN-C immunoreactivity, the only remaining traces of TN-C being in the microtendon part of the MTJ (Fig. 5A). A strong TN-C staining remained in the contralateral MTJs. An identical decrease to that seen in the MTJ was also identified in the expression of TN-C at the MFJ.

Only faint expression of TN-C was detected in the tenocyte-collagen fiber interface of the immobilized Achilles tendons, and no expression was seen around the tendineal collagen fiber bundles (Fig. 5B). Also, the OTJs of the immobilized Achilles tendons showed no or only very mild TN-C expression. In the mRNA in situ hybridization analysis, no expression of TN-C was detected in the immobilized muscle (Fig. 4A), whereas a weak mRNA signal for TN-C was detected in the tendon (Fig. 4D). The healthy contralateral limb had mRNA expression comparable to that in control animals (data not shown).

TN-C expression after free cage activity (Group FR_{11})

The expression pattern of TN-C returned to the same level at the MTJ of the previously immobilized limbs after eight weeks of free remobilization. However, the TN-C expression

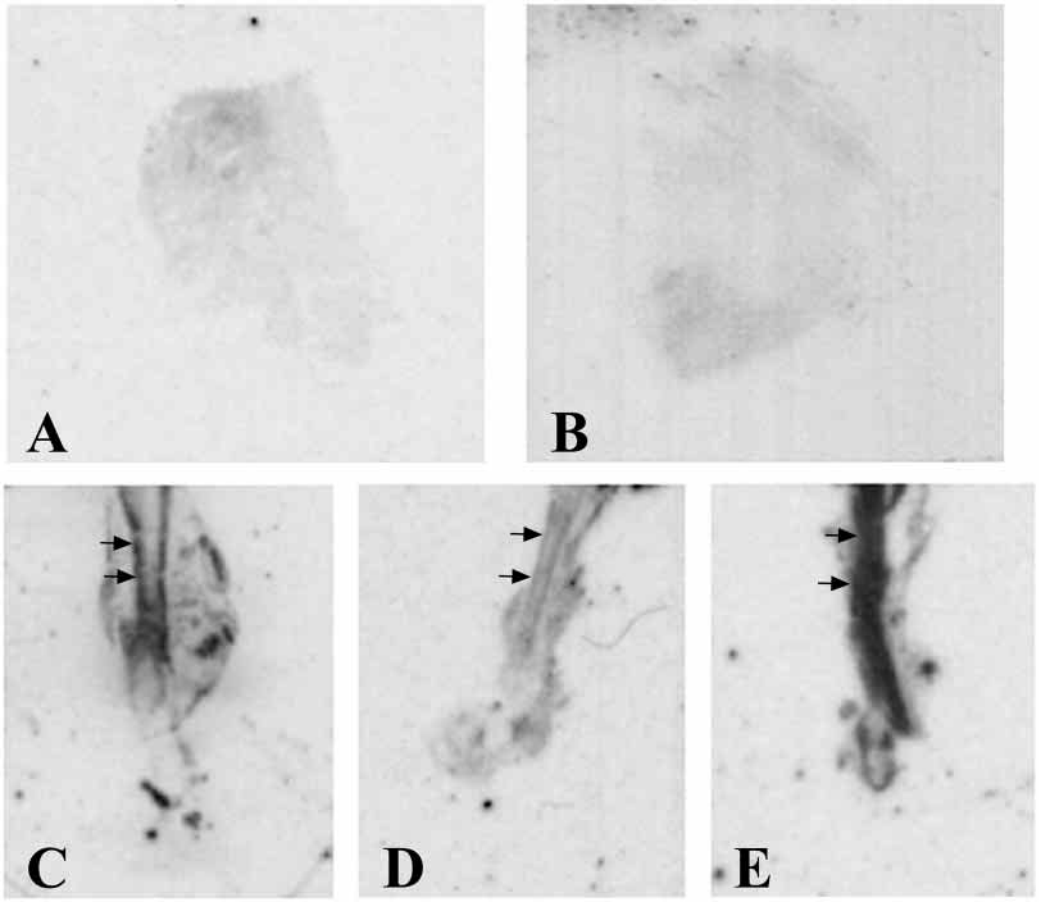

Fig. 4. mRNA in situ hybridization of TN-C. No signal for TN-C mRNA can be detected in the skeletal muscle (cross-sectional section) either (A) after the immobilization or (B) after the high-intensity treadmill running of eight weeks, whereas in the tendon (longitudinal section) (C-E), mechanical loading regulates the mRNA expression of TN-C. (C) A strong signal can be detected from the normal Achilles tendon (longitudinal section), (D) the expression almost disappears after the three-week immobilization, whereas (E) a strong singal for TN-C mRNA is detectable after the high-intensity treadmill running of eight weeks. Magnification ×5.

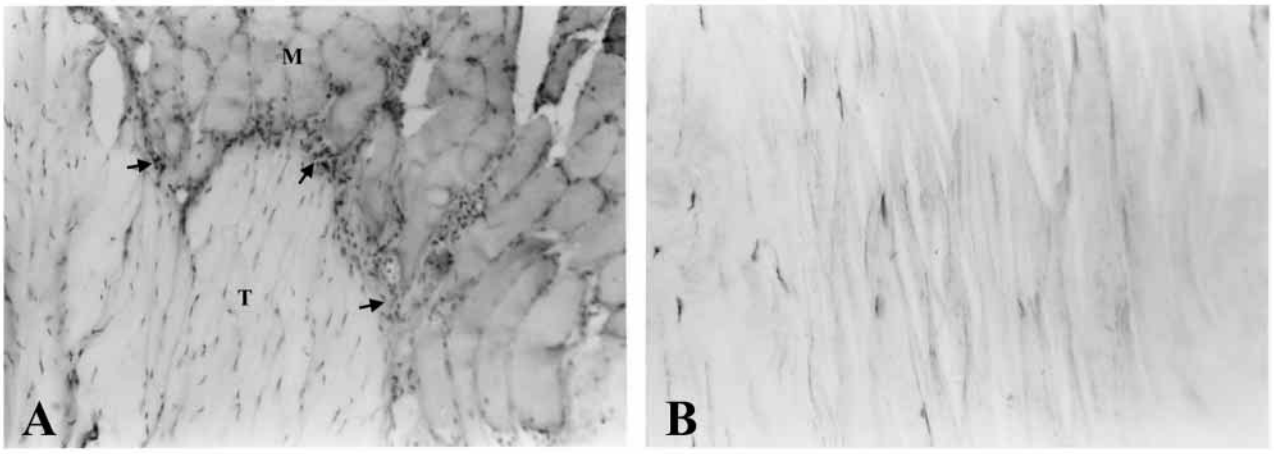

Fig. 5. (A) A marked reduction in the immunohistochemical expression of TN-C can be seen in the MTJ after the three-week immobilization. (B) Only faint TN-C immunopositive signal is visible in the immobilized Achilles tendon after three weeks. Magnification: A,B ×50.

remained at substantially lower levels in the previously immobilized MTJs and Achilles tendons than in the MTJs and Achilles tendons of the contralateral limbs (data not shown). The signal for TN-C did not exceed that of the background in mRNA in situ hybridization analysis of the previously immobilized limbs.

TN-C expression after low- and high-intensity treadmill running (Groups LR_{11} and HR_{11})

There were no visual differences in the TN-C immunoreactivity between the groups of animals remobilized with two different intensive remobilization protocols. A very strong band of TN-C expression was found in both the previously immobilized and the healthy contralateral MTJs of the gastrocnemius muscle, whereas no TN-C immunoreactivity could be detected in the muscle itself (Fig. 6A,B). A strong irregular band of TN-C expresssion was seen also in the myofascial junction of the remobilized animals (Fig. 6A,B).

Large deposits of TN-C were identified in the tenocyte collagen fiber interface and on the surface of the parallel-oriented collagen bundles of the Achilles tendon. mRNA analysis by in situ hybridization confirmed the expression of TN-C in the MFJ, MTJ and in the tendon, both in the previously immobilized and healthy contralateral limbs (Fig. 4E). No TN-C mRNA expression could be detected in the muscle belly after the strenuous remobilization (Fig. 4B).

Discussion

In this study, we have demonstrated that mechanical loading regulates the expression of TN-C at the sites of the muscle-tendon unit normally expressing TN-C (MTJ, MFJ and the tendon). Contrary to a recent study (Flück et al., 2000), even strenuous mechanical loading could not induce the de novo expression of TN-C in the skeletal muscle per se.

Blocking of the normal muscle contractive activity (by cast immobilization) resulted in a marked atrophy of the gastrocnemius muscle, which was not completely reversed even by intensive treadmill running for eight weeks. Even at the end of the remobilization period, the muscles of the previously immobilized limbs showed an increased percentage of muscle fibers with clear atrophic features and an increased amount of intramuscular connective tissue. Despite the

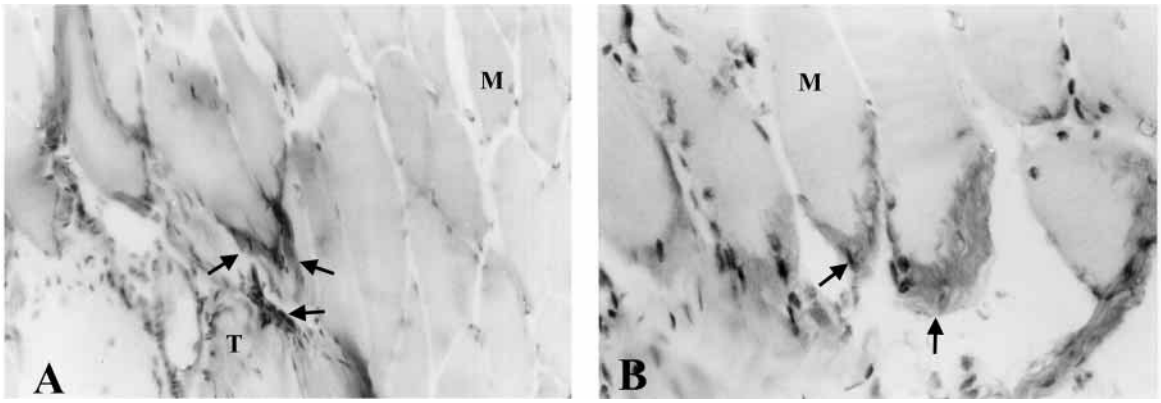

Fig. 6. Strong immunohistochemical expression of TN-C can be seen in the MTJs of both the previously immobilized (A) and the healthy contralateral muscles (B) after the high-intensity treadmill running of eight weeks. The mechanical loading can not induce the de novo expression of TN-C in the previously immobilized nor the healthy contralateral skeletal muscle. Magnification: A,B ×100.

persisting atrophy of the previously immobilized muscles, and, consequently, the atrophied muscle fibers being subjected to higher mechanical stresses than their contralateral (non-immobilized) muscle fibers, we could not detect any TN-C expression in the skeletal muscle after the remobilization period, although the expression of TN-C was regulated by mechanical loading in an apparently dose-dependent fashion in other parts of the muscle-tendon unit.

Our finding is in agreement with previous studies on skeletal muscle showing that TN-C is not expressed in the normal skeletal muscle, except around the blood vessels within the muscle tissue (Hurme and Kalimo, 1992; Irintchev et al., 1993; Settles et al., 1996; Kannus et al., 1998a; Ringelmann et al., 1999; Järvinen et al., 2000). However, in response to such pathological stimuli as trauma to the skeletal muscle, TN-C has been shown to be expressed at the injury site (Hurme and Kalimo, 1992; Irintchev et al., 1993; Settles et al., 1996; Ringelmann et al., 1999). This is especially evident in muscular dystrophies (Gullberg et al., 1997; Settles et al., 1996; Ringelmann et al., 1999; Chen et al., 2000), where the disease itself results in sarcolemmal injury in response to normal muscle contractions and strong, focal expression of TN-C at sites of injury and especially at those sites enriched with inflammatory cells and activated fibroblasts (Settles et al., 1996; Gullberg et al., 1997; Ringelmann et al., 1999; Chen et al., 2000).

However, contrary to all the above-desribed data on the expression of TN-C in the skeletal muscle, Flück et al. recently reported a rapid induction (within 4 hours) of TN-C expression in the skeletal muscle in response to fixing a weight equal to 10% of the total weight of the animal to the latissimus dorsi muscle of the chicken wing (Flück et al., 2000). However, it is worth noting that Flück et al. (Flück et al., 2000) reported an almost 50% increase in the mass of the loaded muscle as soon as 4 hours after the beginning of elongation, concomitant with a marked early infiltration of inflammatory cells (macrophages and neutrophils) and widening of the endomysial spaces in the loaded muscle. It is virtually impossible for a mechanical load to induce an anabolic effect of such magnitude on the synthesis rate of the skeletal muscle proteins that it could explain the rapid and massive increase in the mass of the skeletal muscle (+44% at 4 hours, +42% at 10 hours, +92% at 24 hours after the beginning of loading) as reported by Flück et al. (Flück et al., 2000). The increased muscle mass, the endomysial widening and the massive inflammatory cell reaction are probably attributable to a loading-induced over-extension injury to the skeletal muscle, causing disruption of the intramuscular capillaries and extravasation of inflammatory cells into endometrial spaces of the skeletal muscle. On the basis of the above description, we feel that the TN-C expression reported by Flück et al. is most probably the normal response to tissue injury in the skeletal muscle.

TN-C is enriched at certain locations in the musculoskeletal tissue that are exposed to heavy mechanical loading. In this study we show that in three such sites, the MTJ, the MFJ and the tendon, the prevailing level of mechanical loading (stress) regulates the expression of TN-C. Considering, on one hand, the recently proposed function of TN-C as an elastic protein, and, on the other, the sites where TN-C is expressed, it can be proposed that TN-C provides some elastic properties for the muscle-tendon unit. The myofiber-microtendon interface, which is mechanically the most vulnerable site for injury in the muscle-tendon unit (Kääriäinen et al., 2000a; Kääriäinen et al., 2000b), is the site of highest TN-C expression.

In summary, this study shows that the expression of matricellular protein TN-C is regulated in the muscle-tendon unit by mechanical strain. Our study also shows that mechanical loading alone (without accompanying injury to the tissue) cannot induce de novo synthesis of TN-C in the skeletal muscle. Our results further support the current concept that TN-C provides elasticity for mesenchymal tissues subjected to heavy tensile loading. Thus, we propose that mechanical loading regulates the normal expression of TN-C in the musculoskeletal tissues, but disruption of the mechanical integrity of the tissue is required for the induction of the de novo synthesis of this protein.

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