

Reloading of atrophied rat soleus muscle induces tenascin-C expression around damaged muscle fibers

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Flück, Martin, Matthias Chiquet, Silvia Schmutz, Marie-Hélène Mayet-Sornay, and Dominique Desplanches. Reloading of atrophied rat soleus muscle induces tenascin-C expression around damaged muscle fibers. *Am J Physiol Regul Integr Comp Physiol* 284: R792–R801, 2003; 10.1152/ajpregu.00060.2002.—The hypothesis was tested that mechanical loading, induced by hindlimb suspension and subsequent reloading, affects expression of the basement membrane components tenascin-C and fibronectin in the belly portion of rat soleus muscle. One day of reloading, but not the previous 14 days of hindlimb suspension, led to ectopic accumulation of tenascin-C and an increase of fibronectin in the endomysium of a proportion (8 and 15%) of muscle fibers. Large increases of tenascin-C (40-fold) and fibronectin (7-fold) mRNA within 1 day of reloading indicates the involvement of pretranslational mechanisms in tenascin-C and fibronectin accumulation. The endomysial accumulation of tenascin-C was maintained up to 14 days of reloading and was strongly associated with centrally nucleated fibers. The observations demonstrate that an unaccustomed increase of rat soleus muscle loading causes modification of the basement membrane of damaged muscle fibers through ectopic endomysial expression of tenascin-C.

mechanical stress; fibroblast; extracellular matrix

HOMEOSTASIS OF SKELETAL MUSCLE is controlled by loading states. Generally, unloading causes loss of mass (atrophy) and strength in antigravitational skeletal muscles (23, 29, 36), whereas stimuli increasing skeletal muscle load cause opposite adaptations (1, 22, 30, 59, 69). Although increased mechanical load is known to increase skeletal muscle mass, it can also cause injury of muscle fibers, which triggers a host inflammatory response that is followed by muscle fiber regeneration (6).

Mechanical stress is also an important regulator of the composition of the extracellular matrix (ECM) in many tissues (10, 48). The possible regulation of ECM protein expression by mechanical stress is particularly well illustrated by the distribution of tenascin-C and fibronectin, two modular ECM proteins that are associated with basement membranes (56, 60). Tenascin-C

is expressed where mechanical stress is highest, e.g., in tendon and the myotendinous junction, muscle spindles, and blood vessels (10, 47). Fibronectin, a ubiquitously expressed ECM protein, is enriched at anatomic sites where firm bindings are needed, e.g., between muscle fibers and fiber bundles (42). Moreover, application of tensile stress rapidly induces the expression of tenascin-C, and to a less pronounced extent fibronectin, in cultured fibroblasts (16, 44, 67), and a stretch-responsive enhancer region has been identified in the chicken and human tenascin-C promoter (10, 16). The mechanism by which mechanical stress controls expression of ECM proteins tenascin-C and fibronectin thus may involve directly (immediately) and indirectly activated pathways (46). In this regard, immune reactions and associated cytokines are potential factors that could be released or produced as a consequence of mechanical stress and that could induce tenascin-C and fibronectin expression (32, 33, 46, 55, 68, 72).

Despite their pivotal role in the musculoskeletal system for growth and structural and functional integrity (24, 32, 35, 38, 39), little is understood about the influence of mechanical loading on the expression of ECM proteins in mammalian skeletal muscle (62). Recently, we reported that loading rapidly and reversibly controls the expression of tenascin-C in endomysial fibroblasts of chicken skeletal muscle (27). In this model, within 4 h of loading, ectopic expression of tenascin-C was induced in the belly portion of the anterior latissimus dorsi muscle before signs of macrophage infiltration were apparent, and tenascin-C expression decreased again with removal of the load. This is of potential importance because expression of both tenascin-C and fibronectin correlated with myoblast fusion in culture (38), and both proteins exert, in addition to their structural function and elastic properties (15, 53), adhesive and immunomodulatory activities, thereby influencing repair and adaptation (52) and monocyte/macrophage recruitment (58, 63), respectively.

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Suspension of rat hindlimbs is a widely used model that produces unloading of the antigravitational soleus muscle and permits increasing the activity and gravitational loading of the muscle thereafter (8). As little as 14 days of suspension causes severe atrophy and transformation of slow-twitch to fast-twitch fibers of the soleus muscle (54). Thereafter, when rats are given access to normal cage activity, cross-sectional area and percentage distribution of fiber types in atrophied muscle recover spontaneously (22). Furthermore, hindlimb suspension (3 wk) causes soleus muscle fiber degeneration (7). Reloading of 10- to 12.5-day hindlimb-suspended rats leads, presumably as a consequence of acute fiber damage, to transient infiltration of damaged soleus muscle fibers with macrophages (and monocytes) within the first 2 days (43, 65). With longer duration (>4 days) of reloading the atrophied rat soleus muscle, an increase in regenerating fibers, arising subsequent to fiber damage (6, 7, 20), is observed when the number of invaded muscle fibers has returned to the control level (43, 61). Morphologically, the acute and chronic muscle fiber damage (and eventual regeneration) is indicated by the presence of fibers with central nuclei (50, 61). Hindlimb reloading after 14 days of unloading was associated with preferential damage to slow-twitch fibers in rat adductor longus muscle (70). The factors associated with fiber damage and subsequent fiber infiltration in reloaded soleus muscle could cause increased ECM synthesis (9, 32, 33, 46, 55).

The present investigation aimed to measure the influence of mechanical loading of rat soleus muscle on the spatial-temporal expression of ECM proteins tenascin-C and fibronectin and its relation to damaged fiber types. In particular, we tested the working hypothesis that 1) hindlimb suspension of rat soleus muscle reduces, while subsequent reloading of unloaded rat soleus muscle induces, expression of tenascin-C and fibronectin in the belly portion of the soleus muscle, 2) the synthesized ECM proteins accumulate in the endomysium of reloaded soleus muscle, and 3) endomysial tenascin-C protein expression is associated with signs of muscle damage indicated by the presence of centrally nucleated fibers.

MATERIALS AND METHODS

Antibodies. Rabbit anti-human plasma fibronectin antiserum 1801 was the same as described previously (27). Rabbit antiserum 142 was raised against chick tenascin-C and affinity purified as described (12, 71). Both antisera cross-reacted with the corresponding rat homolog. Tenascin-C was purified by monoclonal antibody (mAb) affinity chromatography from supernatant of primary chicken dermal fibroblasts as described (12). Slow and fast myosin isoform-specific mAbs were from Chemikon (JURO, Lucerne, Switzerland) and Sigma Chemical (Buchs, Switzerland), respectively. Uncoupled and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Cappel (ICN Biomedicals). Mouse anti-peroxidase complex (mPAP) was purchased from Jackson Laboratories (West Grove, PA).

Unloading and reloading of rat skeletal muscle. Hindlimbs of young female Wistar rats were unloaded in individual

cages by hanging the rats by their tail as described (22). One set of animals was hindlimb suspended for 2 wk (HU14). Another set of animals was after 2 wk of hindlimb suspension allowed to perform normal cage activity for 1, 5, or 14 days, therefore permitting reloading of the soleus muscle (HU-R1, HU-R5, and HU-R14). At the end of the hindlimb suspension protocol and the different reloading periods, rats were weighed and anesthetized, and the soleus muscles of both hindlimbs were dissected out. The muscles were immediately weighed, frozen in melting isopentane, cooled in nitrogen, and stored at -80°C . Animals of the same age as the rats before and after hindlimb suspension and after recovery served as experimental controls (C0, C14, and C28). Six animals each were used per time point. The animal experiments were carried out in the animal facilities of the Faculté de Médecine, Université Lyon I in Lyon, France, according to the newest guiding principles for research (2).

RNA extraction and RT-PCR. Total RNA was extracted using the RNeasy minikit (Qiagen AG, Basel, Switzerland) with guanidine isothiocyanate-containing buffer RLT (included in the RNeasy minikit) with a Polytron mixer (Kimateca), the proteins were digested for 1.5 h at 45°C with proteinase K (120 mAU; Qiagen), and the sample was applied to the column. After washing and a DNase I digestion (Qiagen), the RNA was eluted with water. The integrity of the RNA was checked with formaldehyde gels, and total RNA concentration was estimated using the RiboGreen RNA quantification kit (Molecular Probes, JURO, Lucerne, Switzerland). An aliquot corresponding to 600 ng total RNA was reverse transcribed with the Omniscript Reverse Transcriptase kit (Qiagen) using random hexamer primers following the manufacturer's instructions. Real-time PCR amplification reactions were carried out in triplicate on 30- μl aliquots in 96-well plates on an ABI Prism 5700 Sequence Detection System with probe detection via SYBRGreen (PE Biosystems, Rotkreuz, Switzerland). Tenascin-C and 28S primers were designed with the Primer Express software (PE Biosystems) and determined to have at the working dilution of 700 nM a relative efficiency for amplification of tenascin-C, fibronectin, and 28S of 1.95, 2.0, and 1.9, respectively. For tenascin-C (and fibronectin) and 28S PCR, respectively, ~ 5 ng and 0.5 ng cDNA was used per sample. The amount of target (tenascin-C, fibronectin) mRNA relative to the reference (28S) was calculated using the comparative CT (threshold cycle for target amplification) method according to user bulletin 2 of the ABI Prism 7700 Sequence Detection System with the modification that the relative efficiency of each primer was included in the calculation.

SDS-PAGE and immunoblotting. Soluble ECM proteins were extracted from pooled cryosections with cold deoxycholate buffer by repetitive cycles of vortexing and pipetting and were harvested in the supernatant of a centrifugation step (5 min at 5,000 g, 4°C) as described (27). Protein concentration was determined with the bicinchoninic acid assay (Sigma Chemicals), and samples were denatured by diluting to 1 $\mu\text{g}/\mu\text{l}$ in SDS-PAGE loading buffer and heating to 95°C for 5 min. Twenty micrograms of soluble protein each were run on 5% SDS-PAGE, the gel was Western blotted on nitrocellulose membranes (Schleicher & Schuell, Riehen, Switzerland), and blots were stained with Ponceau S (SERVA, Heidelberg, Germany) to verify for equal loading. Immunodetection using rabbit polyclonal tenascin-C or fibronectin antiserum, signal detection with enhanced chemoluminescence (Super Signal West Pico from Pierce, Socochim SA, Switzerland), and recording on film was as described (27). Films were scanned at a resolution of 300×300 dpi and saved under the tag image file format. The sum of pixels for

each band of interest was then estimated with AIDA Array Easy software (Raytest Schweiz, Urdorf, Switzerland). To combine the data from different Western blot experiments, the signal intensities of the band of interest in different samples on the same blot were standardized to the pixel sum measured for the concomitantly separated 14-day control sample. As extraction of ECM protein tenascin-C (and others) from mammalian skeletal muscle tissue is limited (41), exponential changes were additionally quantified on immunohistochemically stained sections (see below).

Immunohistochemistry. Cryosections (12 μm) from the belly portion of the soleus muscle were fixed in 4% paraformaldehyde, tissue peroxidase activity was quenched with 3% H_2O_2 , and sections were blocked in 3% BSA/PBS and incubated with a 1:400 dilution in 0.3% BSA/PBS of polyclonal rabbit antibodies (142 or 1801) specific for tenascin-C or fibronectin, respectively. In some cases, affinity-purified tenascin-C antibody 142 was applied at a 1:50 dilution. After serial washes in PBS, the sections were reacted with peroxidase-conjugated anti-rabbit IgG (1:2,000 in 0.3% BSA/PBS), again washed in PBS; immunoreactivity was detected with substrate 3-amino-9-ethylcarbazole (Sigma Chemicals, Buchs, Switzerland), and nuclei were counterstained with hematoxylin. To reduce background staining, detection of tenascin-C and fibronectin, respectively, was stopped after 20 and 5 min. For each experiment a control reaction with the same concentration of normal serum as the first antibody was carried out. Alternatively, affinity-purified tenascin-C antibody (protein concentration of 20 $\mu\text{g}/\text{ml}$), which was incubated overnight in PBS containing a fivefold excess of purified chicken tenascin-C protein or BSA and which was centrifuged to remove formed antibody-tenascin-C complexes, was used as a control.

For detection of fiber types, sections were pretreated as described above, followed by sequential incubation with monoclonal slow or fast myosin-specific antibody (1:100 in 0.3% BSA/PBS), then goat anti-mouse whole IgG (1:500), followed by mPAP (1:5,000) with intermittent washing steps in PBS. Immunoreactivity and nuclei were visualized as indicated above.

Quantification of immunohistochemical ECM protein signal. For each (left) muscle soleus from four to six individual animals, one section from the belly portion was stained. Micrographs, each displaying on average 75 fibers, were taken from randomly chosen and nonoverlapping fields of the sections stained for tenascin-C and fibronectin and recorded on slide film (Ekrachrome 64T, Kodak). The photographs were projected at a final magnification of 1:200 on a white screen. The number of all fibers as well as the number of intense immunoreactive endomysia in each projection was then counted. Typically, 400 fibers were counted from each muscle section. For each muscle section, the percentage of immunoreactive endomysia over the counted fibers was calculated. Then for each treatment, the mean percentage and SE of immunoreactive endomysia for the values from the sections of the individual animals were calculated.

Association of centrally nucleated fibers and endomysial tenascin-C expression, respectively, was determined in a similar manner. The total number of muscle fibers, centrally nucleated fibers, and endomysial tenascin-C-positive (or -negative) fibers showing central nuclei was counted from projections of photographs from randomly chosen fields of tenascin-C-stained sections. Fibers were assigned as centrally nucleated based on the presence of hematoxylin-stained nuclei inside the fiber in compliance with earlier definitions (57). Typically, 400 fibers were counted per mus-

cle section, and muscles from four individual animals were analyzed per treatment.

Association of endomysial tenascin-C expression to fiber types was detected in the same manner from consecutive sections of reloaded soleus muscles stained for tenascin-C and slow and fast myosin heavy chain, respectively. Fibers were typed according to published criteria (22).

Data analysis. The percentage of tenascin-C- or fibronectin-positive endomysia, the tenascin-C and fibronectin (pixel) signals in Western blots, as well as the tenascin-C/28S and fibronectin/28S mRNA ratios, were statistically analyzed for an effect of treatment using a one-way ANOVA. Post hoc analyses were performed using a least significant difference test. Statistical analyses were completed using Statistica software [version 5.1 for Windows, StatSoft (Europe), 20253 Hamburg, Germany].

Significance of association of centrally nucleated fibers to endomysial tenascin-C was verified in the following way. For each animal from the reloading groups, two \times two contingency tables of endomysial tenascin-C immunoreactivity [tenascin-C negative (tn-), tenascin-C positive (tn+)] vs. fiber character [centrally nucleated (cn+), not centrally nucleated (cn-)] of soleus muscles were constructed. Independence of the two categories (endomysial tenascin-C immunoreactivity, fiber character) was verified with *G*-test (Frequency Matrix Applet version 2.1, <http://caspar.bgsu.edu/~software/Java/1Contingency.html>). The null hypothesis for a contingency table (cn+ = cn-) was rejected at the $P \leq 0.05$ level. For the unloading and each reloading treatment, the average percentages and SE of cn+/tn+ fibers and cn+/tn- fibers were calculated, and the averages were tested for significant differences using the Kruskal-Wallis *H* test at the $P \leq 0.05$ level using Statistica software. Subsequently, association of endomysial tenascin-C to centrally nucleated fibers was verified in the same manner.

The significance of an association of tenascin-C to slow-, intermediate-, or fast-fiber type was verified analogously with the modification that for each animal from the different reloading groups, two \times three contingency tables of endomysial tenascin-C immunoreactivity (tn-, tn+) vs. fiber type (slow type, intermediate type, fast type) were constructed for the *G*-test.

RESULTS

Reciprocal changes in soleus muscle mass with unloading and reloading. Soleus muscle wet weight was significantly reduced by 47% after 14 days of unloading. Reloading caused an increase in soleus muscle weight, and recovery of mass was complete within 5 days of reloading (Fig. 1). The ratio of soleus muscle weight to body mass was similarly affected.

Tenascin-C and fibronectin accumulation in reloaded soleus muscle. To detect fibronectin and tenascin-C in rat soleus muscle, we verified the specificity of available antiserum with immunoblotting technique from extracts of the belly portion of the muscle. These experiments indicated that small tenascin-C splice variants, 190–200 kDa in size (14), are present at a detectable level in control rat soleus muscles (Fig. 2). Fibronectin was detected as a single band of 220 kDa (Fig. 2). Relative to age controls, no significant change in fibronectin and tenascin-C abundance in 14-day unloaded soleus muscle was found, while reloading caused an increase in fibronectin and tenascin-C vari-

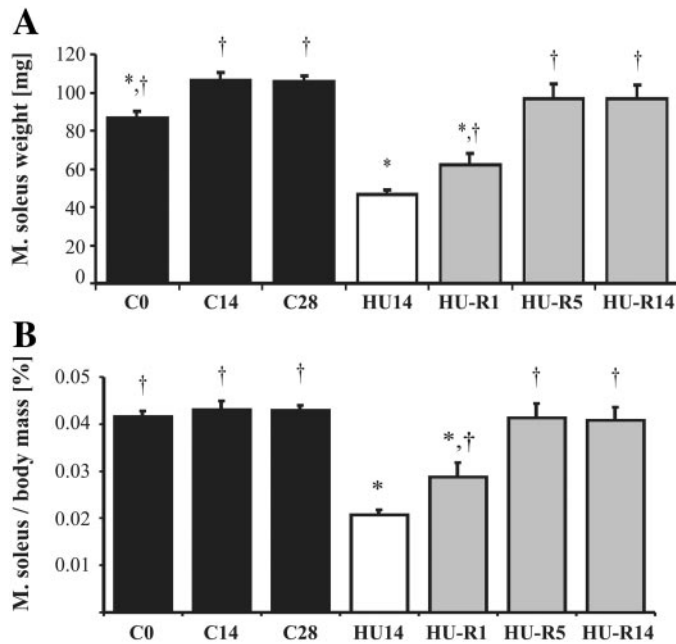


Fig. 1. Effect of unloading and reloading on soleus muscle wet weight. Mean and SE of soleus muscle wet weight (A) and the soleus muscle-to-body mass ratio (B) of rats after 2 wk of unloading by hindlimb suspension (HU14) and subsequent reloading for 1, 5, or 14 days (HU-R1, HU-R5, HU-R14). Animals of the age of the rats before (C0) and after they were subjected to unloading (C14) and reloading (C28), respectively, served as age controls. * $P < 0.05$, significant difference vs. age controls C14 and C28. † $P < 0.05$, significant difference vs. HU14 ($n = 6$). Unloading causes severe atrophy of soleus muscle, which regenerates with reloading.

ants in soleus muscle extracts ($P < 0.05$; Fig. 2). For the 1-day reloading duration, however, the increase in tenascin-C was not significant. Small (190–200 kDa) and large (240–250 kDa) tenascin-C splice variants and a 170-kDa tenascin-C fragment were present in reloaded soleus muscles (13, 14) (Fig. 2).

Accumulation of tenascin-C and fibronectin in the endomysial compartment of reloaded soleus muscle. We pursued quantitative analysis of tenascin-C and fibronectin abundance in rat soleus muscle with immunohistochemical techniques to verify the alteration in ECM protein abundance observed in immunoblotting experiments.

Tenascin-C expression (accumulation) in the belly portion of control soleus muscle was restricted to blood vessels and nerves (Fig. 3). In unloaded soleus muscles, rare tenascin-C accumulation (<0.5% of total fibers) was detected but was not significantly different from controls (Figs. 3 and 5).

Reloading of previously unloaded soleus muscle caused a marked ectopic induction of tenascin-C in the endomysium in the belly portion of the muscle (Fig. 4). Occasionally, tenascin-C-positive endomysial cells were detected in reloaded soleus muscles (see Fig. 4). Control reactions with normal rabbit antiserum and tenascin-C antiserum 142 that had been absorbed to purified chicken tenascin-C demonstrated the specificity of the staining for tenascin-C (Fig. 4). Quantitative analysis revealed that as early as after 1 day of reload-

ing, the abundance of tenascin-C in the endomysium of the middle portion of the soleus muscle increased from a very low level to 8.0% of fibers (Fig. 5A). The endomysial accumulation of tenascin-C was clustered to distinct bundles and stayed elevated at a similar level up to 14 days of reloading (Figs. 4 and 5).

Fibronectin protein in control soleus muscles was mostly confined to large and small blood vessels, nerves, and spindles and was occasionally found in the endomysium (Fig. 3). Relative to the age control, reloading for 1 day caused an increase in the percentage of fibronectin-positive endomysia in soleus muscles from 2.1 to 15.0% (Fig. 5B).

Tenascin-C and fibronectin mRNA. The pronounced endomysial accumulation of tenascin-C and fibronectin in reloaded soleus muscles prompted us to analyze the tenascin-C expression in more detail. To identify to what extent transcriptional events contribute to the increase in tenascin-C, RT-PCR experiments were carried out. These experiments revealed that tenascin-C mRNA (per 28S rRNA) was increased >40-fold relative to the age control after 1 day of reloading (Fig. 6A). After 5 days of reloading, the level of tenascin-C mRNA was reduced and not significantly different from the level of control soleus muscles.

Fibronectin mRNA relative to age control was not affected with unloading but was increased sevenfold by subsequent 1 day of reloading (Fig. 6B). With longer duration of reloading the concentration of fibronectin mRNA was not different from control.

Endomysial tenascin-C is associated with centrally nucleated fibers of reloaded soleus muscle. We pursued experiments to determine whether the distinct tenas-

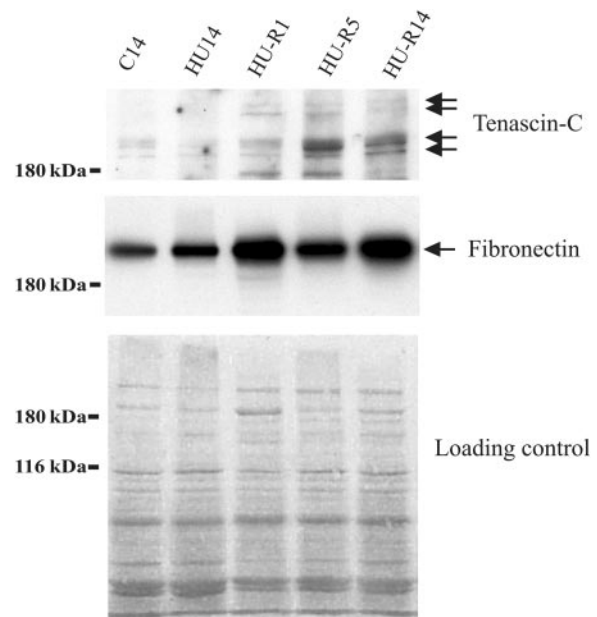


Fig. 2. Tenascin-C and fibronectin abundance: immunoblotting analysis for tenascin-C variants (top, arrows) and fibronectin (middle, arrow) in extracts of rat soleus muscle subjected to different loading protocols. The loading control is shown at bottom. Molecular mass markers are indicated at left. Reloading induces tenascin-C and fibronectin accumulation.

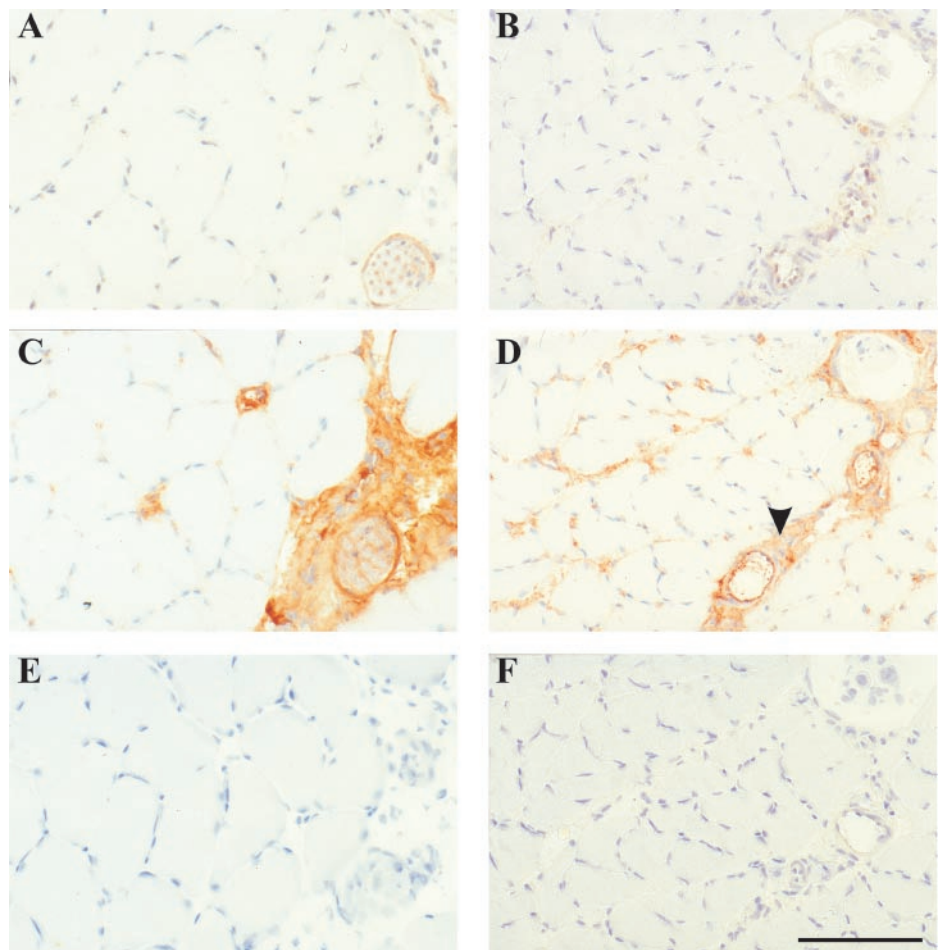


Fig. 3. Tenascin-C and fibronectin in unloaded soleus muscle. Immunohistochemical analysis of tenascin-C (A and B) and fibronectin (C and D) in cryosections from the belly portion of 14-day control (A, C, and E) and 14-day unloaded (B, D, and F) rat soleus muscle. Positive immunostaining is orange, and nuclei appear in blue. Additionally, negative control reaction with normal rabbit serum was carried out (E and F). Arrowhead denotes a fibronectin-positive perivascular structure. Bar, 100 μ m. Unloading does not modify the localization of tenascin-C and fibronectin.

cin-C immunoreactivity is associated with centrally nucleated fibers or to a particular fiber type (Fig. 4, I–K). Relative to their high occurrence, type I fibers did not show endomysial tenascin-C more frequently than type II fibers in 1-day reloaded soleus muscles ($P = 0.6$ for the G -test of contingency tables and Kruskal-Wallis H test).

Centrally nucleated fibers were equally present in 14-day unloaded and 1-day reloaded soleus muscles and increased in 5- and 14-day reloaded soleus muscles

(lane “cn+/total” in Table 1). The mean percentage of centrally nucleated fibers in 14-day unloaded soleus muscles was higher than the one in control soleus muscles, although this difference did not reach statistical significance ($P = 0.10$). A proportion of endomysial tenascin-C-positive fibers in sections of unloaded and reloaded soleus muscles was centrally nucleated (Table 1). This percentage of endomysial tenascin-C-positive soleus muscle fibers having central nuclei (cn+/tn+) was significantly different from the fre-

Table 1. Endomysial tenascin-C accumulation in centrally nucleated fibers

Fibers	Treatment				
	C14	HU14	HU-R1	HU-R5	HU-R14
cn+/total	0.1 \pm 0.1%	0.5 \pm 0.2%	0.6 \pm 0.1%	3.3 \pm 1.1%*†	2.6 \pm 0.9%*†
cn+/tn+	25.0 \pm 9.5%	70.1 \pm 5.9%	14.5 \pm 7.8%	29.7 \pm 0.4%	29.3 \pm 5.4%
cn+/tn-	0.0 \pm 0.0%	0.2 \pm 0.1%	0.04 \pm 0.04%	0.6 \pm 0.4%	0.6 \pm 0.1%
tn+/cn+	100 \pm 0.0%	84.5 \pm 9.0%	95.0 \pm 5.0%	87.8 \pm 6.5%	69.5 \pm 9.4%
tn+/cn-	0.2 \pm 0.1%	0.5 \pm 0.2%	7.5 \pm 2.9%	6.9 \pm 2.1%	4.2 \pm 1.0%

Values are means \pm SE. The mean percentage of centrally nucleated fibers and association of tenascin-C-positive endomysia with centrally nucleated fibers in reloaded soleus muscles were determined from 4–6 animals as described in MATERIALS AND METHODS. tn+ and tn-, tenascin-C-positive or -negative fibers, respectively; cn+ and cn-, fibers containing or lacking central nuclei, respectively. Treatment groups: rats after 2 wk of unloading by hindlimb suspension (HU14) and subsequent reloading for 1, 5, or 14 days (HU-R1, HU-R5, HU-R14); C14, control rats of the age of rats after they were subjected to unloading. *Significant difference from C14 (ANOVA, $P < 0.05$). †Significant difference from HU14 (ANOVA, $P < 0.05$). G -test indicated in all individual samples, except for the C14 group, significant independence ($P < 0.05$) of categories for contingency tables of endomysial tenascin-C immunoreactivity and fiber character (appearance of central nuclei). ‡Significant difference ($P < 0.05$) between average values from same treatment as calculated with Kruskal-Wallis H test.

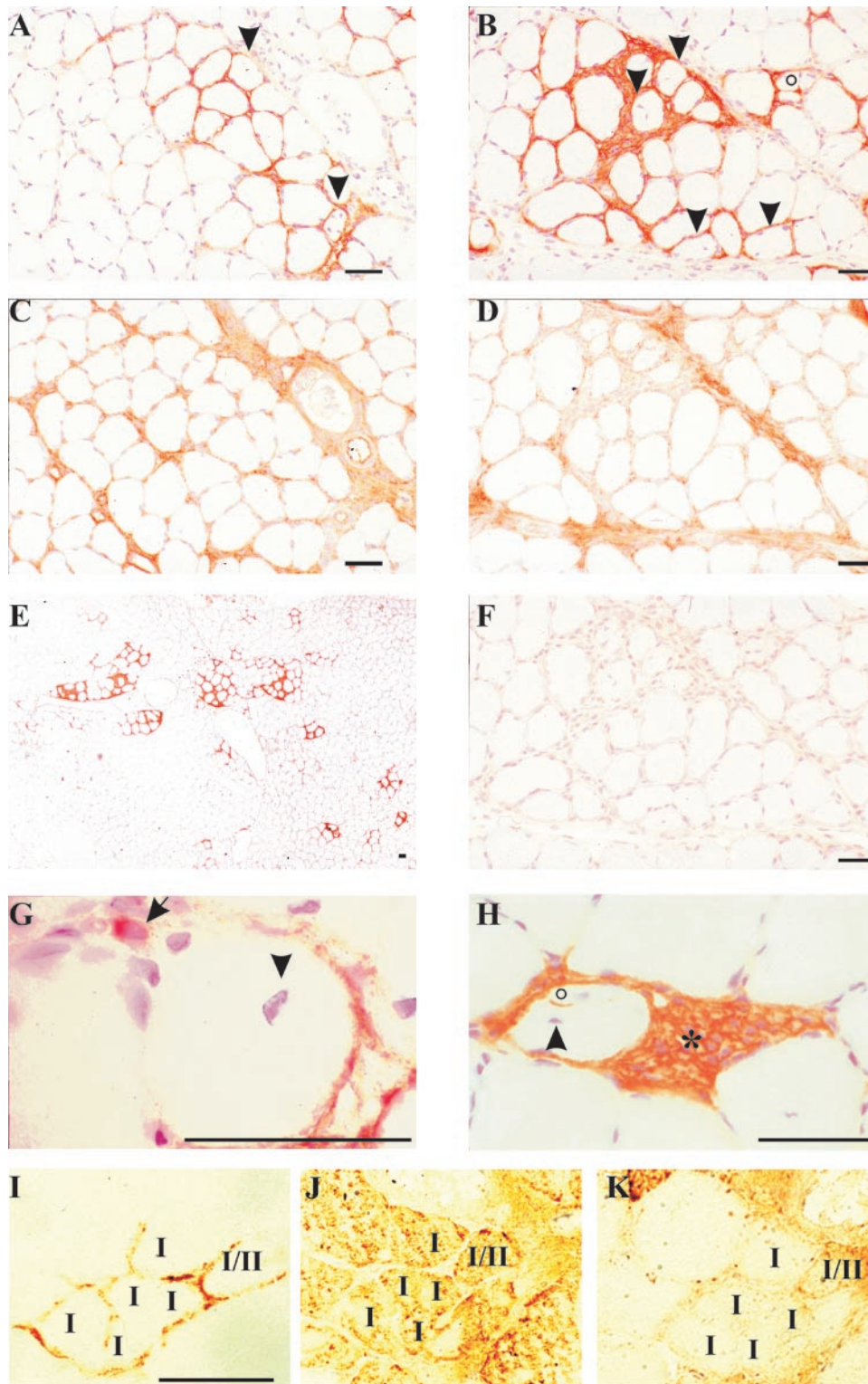


Fig. 4. Tenascin-C and fibronectin in reloaded soleus muscle. A–F: micrographs from immunohistochemical analysis of tenascin-C (A, B, and E) and fibronectin (C and D) in cryosections of 1-day (A and C) and 5-day (B, D–F) reloaded rat soleus muscle. The micrograph shown in E appears at a lower magnification. Positive immunostaining is orange, and nuclei appear in blue. F: negative control reaction with tenascin-C antiserum after its absorption to purified tenascin-C demonstrates specificity of tenascin-C staining. G and H: higher magnified micrographs showing tenascin-C immunostaining of fibers with central nuclei (arrowhead; see also A and B), a split fiber (circle; see also B), and an invaded fiber (*) in a 1-day (G) and 14-day (H) reloaded soleus muscle. Arrow indicates a tenascin-C-producing endomysial cell. I–K: descriptive immunohistochemistry for tenascin-C (I) and type I (J) and type II (K) myosin heavy chain. I and II denote different muscle fiber types. Bar, 50 μ m. Reloading causes ectopic accumulation of tenascin-C in the endomysial compartment of certain bundles.

quency of tenascin-C-negative fibers containing central nuclei (cn+/tn-), whatever the experimental treatment (unloading or reloading) (Table 1). The percentage of central nuclei per endomysial tenascin-C-positive fibers (cn+/tn+) was higher in unloaded than in loaded soleus muscles. The majority (≥ 69.5) of centrally nucleated fibers in unloaded and reloaded soleus

muscles showed endomysial tenascin-C accumulation (lane "tn+/cn+" in Table 1; see Fig. 4). Compared with the frequency of non-centrally nucleated fibers positive for tenascin-C (tn+/cn-), the differences in the percentage of centrally nucleated fibers showing tenascin-C accumulation (tn+/cn+) were significant. The majority of central nuclei in endomysial tenascin-C-

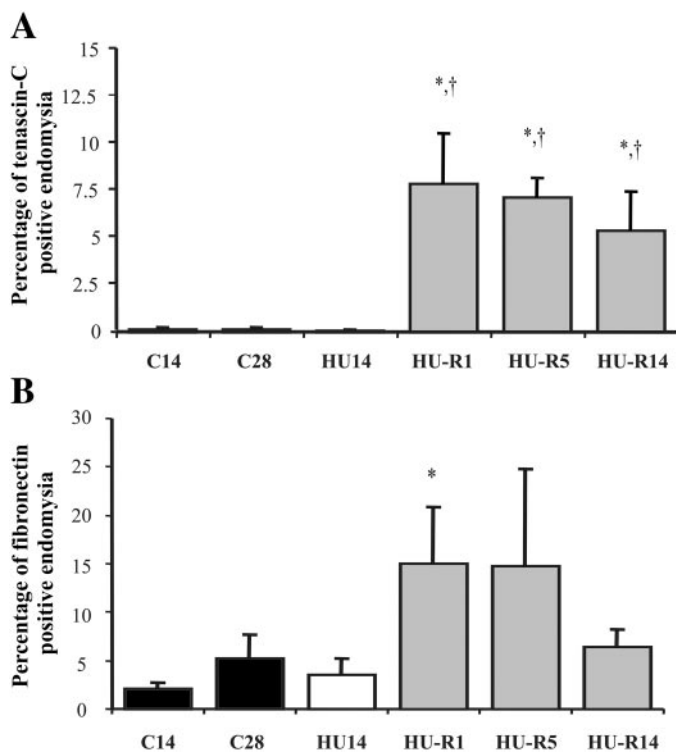


Fig. 5. Quantification of endomysial tenascin-C and fibronectin accumulation. Mean and SE of endomysial tenascin-C (A) and fibronectin (B) accumulation in rat soleus muscle subjected to different loading protocols. * $P < 0.05$, significant difference vs. respective age control C14 or C28. † $P < 0.05$, significant difference vs. HU14 ($n = 4-6$ for each time point). For abbreviations, see legend to Fig. 1. Within 1 day of reloading, tenascin-C and fibronectin accumulate in the endomysium of a proportion of soleus muscle fibers.

positive fibers of 1- and 5-day reloaded soleus muscles were compact (Fig. 4G). In addition, rare tenascin-C accumulation was detected in split fibers and infiltrated fibers of reloaded soleus muscles (Fig. 4, B and H).

DISCUSSION

Changes in mechanical loading are important physiological stimuli causing adaptations of skeletal muscle mass. A main focus of previous research concerning the adaptations of mammalian skeletal muscle to changes in muscle loading was on contractile (34, 45), metabolic (22, 25, 64, 66), and cytoskeletal proteins (17, 28). Here we show for the first time that expression of the two ECM proteins, tenascin-C and fibronectin, in anti-gravitational rat skeletal muscle is loading dependent. While unloading produced no significant trend, expression of tenascin-C and fibronectin mRNA and abundance of tenascin-C variants and fibronectin in the endomysium (of fibers) in certain bundles is rapidly induced with reloading of previously unloaded soleus muscle. Moreover, the endomysial tenascin-C deposition was associated with centrally nucleated muscle fibers.

One day of reloading already caused the ectopic appearance of tenascin-C protein in the endomysium of

8% of fibers in soleus muscles. The net increase in tenascin-C-positive endomysia, relative to the percentage of immunoreactive endomysia in control (C14) and unloaded (HU14) soleus muscles, was similar to the one observed for fibronectin. The increase in tenascin-C and fibronectin in reloaded soleus muscles as detected by immunohistochemistry was confirmed by the observation made on abundance of fibronectin and tenascin-C variants by immunoblotting analysis, except for 1 day of reloading, where the marked increase in endomysial tenascin-C could not be demonstrated with this method. The latter observation supports the contention that extraction of ECM protein tenascin-C (and others) from mammalian skeletal muscle tissue is limited (41). The fact that tenascin-C and fibronectin were not reduced with unloading was paralleled by a nonsignificant difference ($P > 0.7$) of mRNA for tenascin-C and fibronectin mRNA between control and unloaded soleus muscles (Figs. 5 and 6).

Because tenascin-C is normally absent in the endomysia of control muscle (Fig. 3; Ref. 27), the pronounced (ectopic) endomysial accumulation of tenascin-C suggests that tenascin-C expression is de novo induced in the endomysium of reloaded soleus muscle. A substantial (>40-fold) increase of tenascin-C mRNA level after 1 day of reloading (Fig. 6) supports that transcriptional mechanisms greatly contribute to the observed en-

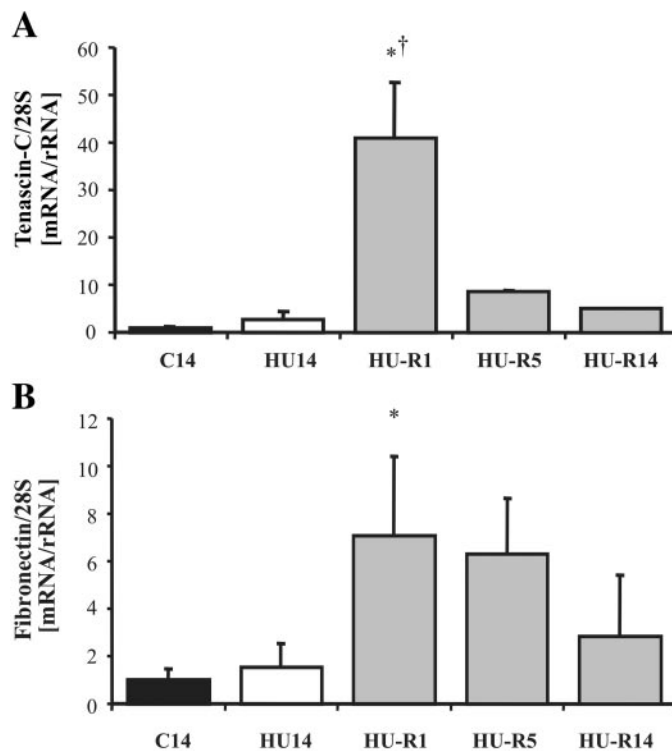


Fig. 6. Quantification of tenascin-C and fibronectin transcripts. Mean and SE of tenascin-C (A) and fibronectin (B) mRNA level in rat soleus muscle subjected to different loading protocols. The ratio of tenascin-C to 28S mRNA and of fibronectin to 28S mRNA in the control C14 was set to 1. *Significant difference vs. age control C14; †significant difference vs. HU14. For abbreviations, see legend to Fig. 1. Tenascin-C and fibronectin mRNA are substantially induced with 1 day of reloading.

endomysial increase in tenascin-C. Similarly, a sevenfold increase in fibronectin mRNA level was detected after 1 day of reloading matching the observed increase in fibers showing endomysial fibronectin protein (compare Figs. 5B and 6B). The fact that only a proportion of fibers did show fibronectin immunoreactivity in control muscle, while it is reported to exhibit a thin endomysial distribution (Fig. 3) (15, 31), may be related to differences in the threshold of detection. The ECM protein tenascin-C and fibronectin are synthesized primarily by fibroblasts but not in muscle fibers (5, 11, 13), and we observed an even faster induction of tenascin-C gene transcription to occur in endomysial fibroblasts of overloaded chicken skeletal muscle (27). In reloaded soleus muscles, endomysial cells were identified as the origin of tenascin-C production (see Fig. 4). Therefore, the endomysial tenascin-C (and fibronectin) accumulation provoked by reloading is likely mediated to a substantial extent by transcriptional activation of the tenascin-C (and fibronectin) gene(s) in endomysial fibroblasts.

Reloading of hindlimb-suspended rats augments the activity and loading of the antigravitational soleus muscle (8). Normal and 14-day hindlimb-suspended rat soleus muscles are primarily composed of slow and fast fatigue-resistant motor units (54), which are frequently recruited during free movement as well as during quiet postural standing (3, 37). Therefore with reloading of 14-day atrophied rat soleus muscles, most muscle fibers are expected to experience significant increases in mechanical stress due to increased force transmission via a serial myotendinous pathway to the tendon and via a lateral myofascial pathway to the connective tissue and adjacent muscle fibers (39). This could affect tenascin-C gene expression directly, i.e., in a cell-autonomous way (10), or may induce damage that could indirectly affect tenascin-C expression (46). The pronounced endomysial tenascin-C accumulation in only a proportion of reloaded soleus muscles therefore indicates a major involvement of indirect pathways, in addition to eventual direct mechanical factors, in the upregulation of tenascin-C expression.

This conclusion is supported by the observation that endomysial tenascin-C accumulation in reloaded soleus muscle was not preferentially attributed to the slow-type fibers that are most frequently recruited during free movement as well as during quiet postural standing (3, 37). On the other hand, the significant association of endomysial tenascin-C accumulation with centrally nucleated muscle fibers (see Table 1) indicates that damage-related factors contribute to the mechanism inducing tenascin-C expression in reloaded soleus muscle (32). Central nuclei in fibers of formerly hindlimb-suspended soleus muscles reflect fibers that are damaged as a consequence of reloading (4, 43, 50). The association of tenascin-C with centrally nucleated fibers is further supported by the observation that a higher percentage of the rare centrally nucleated fibers in unloaded soleus muscles was tenascin-C positive (see Table 1). The appearance of such damaged fibers in soleus muscles with 14 days of unloading also re-

lates to the unchanged level of tenascin-C mRNA relative to control soleus muscles at that time. It is possible that small decreases in staining for tenascin-C around muscle spindles and blood vessels (10, 47) were offset by small increases in staining for tenascin-C around damaged fibers. Alternatively, the differences may be too small to be detected by the employed methodology. Overall, the findings imply that induced endomysial accumulation of tenascin-C and increased tenascin-C mRNA relate to damage of atrophied muscle fibers with reloading.

Autocrine or paracrine action of growth factors, hormones, and cytokines (21, 46, 55) has been implied in upregulation of ECM biosynthesis after tissue damage (32). Within 2 h after reloading of atrophied rat soleus muscle, membrane injury is observed (43, 65). This damage and the fact that the fibers with endomysial accumulation of tenascin-C are clustered to distinct bundles (see Fig. 4E) argue for a local action/production of factors in certain bundles, capable of inducing tenascin-C and fibronectin expression, as a consequence of soleus muscle reloading. In this context it has been demonstrated that the proteins creatine kinase and fibroblast growth factor 2 (FGF2) are released from skeletal muscle cells in culture and in vivo as a consequence of membrane damage caused by mechanical loading and exercise, respectively (18, 19, 49). Microarray analysis demonstrated that from the several cytokines and growth factors known to induce tenascin-C transcription in fibroblasts and to be expressed in muscle fibers (19, 51, 55, 68, 72), two, insulin-like growth factor II (IGF-II) and FGF2, are expressed in control and unloaded soleus muscles used in this study (26). However, their mRNA level was not different between 14-day unloaded and 1-day reloaded soleus muscles (26). Therefore, an early release of soluble factors, two candidates of which are IGF-II or FGF2, from fibers injured due to soleus muscle reloading (65) may link to ectopic tenascin-C expression in the endomysium.

The present data are compatible with the hypothesis that deposition of tenascin-C in endomysia of reloaded rat soleus muscle may modulate the biological and mechanical properties of the basement membrane of atrophied fibers damaged due to an unaccustomed increase in mechanical stress. Tenascin-C is thought to have many functions besides its structural role in stabilizing tissue integrity (15, 35). Tenascin-C causes cellular deadhesion, thereby inducing transition of cells into an intermediate adhesive state that is considered to be an adaptive condition facilitating expression of genes involved in repair and adaptation (52). Expression of tenascin-C correlates with myoblast fusion (38), and an increased level of tenascin-C was observed on the outer surface of regenerating myofibers in skeletal muscle injured by a standardized trauma or motor-driven downhill running (40, 41). Moreover, tenascin-C exerts immunomodulatory activities by modulating monocyte/macrophage recruitment (63), and increased tenascin-C immunoreactivity invariably correlated with the presence of macrophages

(33). In our study, endomysial accumulation of tenascin-C in unloaded and reloaded soleus muscles was associated with centrally nucleated fibers (see Table 1, Fig. 4, arrowheads), which may reflect internalized myonuclei of regenerating fibers or may indicate infiltrating immune cells (7, 50, 61). Moreover, tenascin-C accumulation was observed in occasionally infiltrated and split muscle fibers of 5- to 14-day reloaded soleus muscles (Fig. 4, B and H). Future studies are necessary to conclude the definitive role of endomysial tenascin-C accumulation in the process of muscle fiber infiltration and/or regeneration in reloaded soleus muscles.

Our results support the notion that changes in expression of ECM proteins may play a role early in the regenerative and/or inflammatory events induced in skeletal muscle due to an unaccustomed increase of mechanical loading of damaged muscle fibers. The results indicate the possibility that deposition of tenascin-C is a marker for substantial, i.e., damaging, increases in mechanical stress to skeletal muscle fibers and bundles.

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