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Rapid and reciprocal regulation of tenascin-C and tenascin-Y expression by loading of skeletal muscle

Martin Flück*, Vildan Tunç-Civelek and Matthias Chiquet

M. E. Müller-Institute for Biomechanics, Murtenstrasse 35, PO Box 30, CH-3010 Bern
*Author for correspondence (e-mail: flueck@mem.unibe.ch)

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SUMMARY

Tenascin-C and tenascin-Y are two structurally related extracellular matrix glycoproteins that in many tissues show a complementary expression pattern. Tenascin-C and the fibril-associated minor collagen XII are expressed in tissues bearing high tensile stress and are located in normal skeletal muscle, predominantly at the myotendinous junction that links muscle fibers to tendon. In contrast, tenascin-Y is strongly expressed in the endomysium surrounding single myofibers, and in the perimysial sheath around fiber bundles. We previously showed that tenascin-C and collagen XII expression in primary fibroblasts is regulated by changes in tensile stress. Here we have tested the hypothesis that the expression of tenascin-C, tenascin-Y and collagen XII in skeletal muscle connective tissue is differentially modulated by mechanical stress in vivo.

Chicken anterior latissimus dorsi muscle (ALD) was mechanically stressed by applying a load to the left wing. Within 36 hours of loading, expression of tenascin-C protein was ectopically induced in the endomysium along the surface of single muscle fibers throughout the ALD, whereas tenascin-Y protein expression was barely affected. Expression of tenascin-C protein stayed elevated after 7 days of loading whereas tenascin-Y protein was reduced. Northern blot analysis revealed that tenascin-C mRNA was induced in ALD within 4 hours of loading while tenascin-Y mRNA was reduced within the same period. In situ hybridization indicated that tenascin-C mRNA induction after 4 hours of loading was uniform throughout the ALD muscle in endomysial fibroblasts. In contrast, the level of tenascin-Y mRNA expression in endomysium appeared reduced within 4 hours of loading. Tenascin-C mRNA and protein induction after 4-10 hours of loading did not correlate with signs of macrophage infiltration. Tenascin-C protein decreased again with removal of the load and nearly disappeared after 5 days. Furthermore, loading was also found to induce expression of collagen XII mRNA and protein, but to a markedly lower level, with slower kinetics and only partial reversibility. The results suggest that mechanical loading directly and reciprocally controls the expression of extracellular matrix proteins of the tenascin family in skeletal muscle.

Key words: Mechanical stress, Tenascin, Extracellular matrix, Skeletal muscle

INTRODUCTION

There is ample reason to believe that the amount and composition of extracellular matrix (ECM) is controlled in part by the manner and magnitude of mechanical stress acting on the cells within connective tissue (Lambert et al., 1992; Langholz et al., 1995; Chiquet, 1999). Tenascin-C, an ECM component modulating cell adhesion (Chiquet-Ehrismann et al., 1988), and the minor fibril-associated collagen type XII (Koch et al., 1995) illustrate a possible regulation of ECM protein expression by mechanical stress particularly well. We reported previously that tenascin-C and collagen XII mRNA and protein are reversibly induced by tensile stress in vitro in dermal fibroblasts cultured on a collagen I matrix (Chiquet-Ehrismann et al., 1994; Trächslin et al., 1999). In addition, cyclic stretching of cardiac myocytes (Yamamoto et al., 1999) and of vascular smooth muscle cells (Feng et al., 1999) attached to silicon membranes upregulates tenascin-C expression. In the promoters of both the *tenascin-C* and the *collagen XII* genes, stretch-responsive enhancer regions have been identified (Chiquet-Ehrismann et al., 1994; Chiquet et al., 1998). Interestingly, in vivo tenascin-C and collagen XII are coexpressed in connective tissues subjected to high tensile stress such as tendons, ligaments, periosteum and smooth muscle (Chiquet and Fambrough, 1984; Koch et al., 1995). Indeed, several published reports indicate that these two ECM proteins may also be regulated in vivo, by external mechanical load. So far, however, the only tissues that have been studied are those which normally express high amounts of tenascin-C (arterial walls, periosteum, osteotendinous junction; Mackie et al., 1992; Webb et al., 1997; Järvinen et al., 1999) or collagen XII (periodontal ligament; Karimbux and Nishimura, 1995). Moreover, changes in mRNA and protein levels were observed in these studies after prolonged periods of time, i.e. after one to several days. For these experiments, it could therefore be argued that the effect of

mechanical loading on the changed expression of ECM proteins was indirect, i.e. as a consequence of increased cell proliferation or stress-induced inflammation (Gullberg et al., 1998).

To investigate the hypothesis that tenascin-C or collagen XII expression might be upregulated under the direct control of tensile stress acting on connective tissue cells in vivo, we selected a tissue with low endogenous expression of the two ECM proteins, and investigated early time points after application of a mechanical load. This model should also allow us to correlate increases in ECM expression with the appearance of immune cells, in order to distinguish between direct effects of loading and possible signs of an inflammatory reaction. Skeletal muscle seemed suitable since both tenascin-C and collagen XII are not normally produced by fibroblasts in the endomysium along muscle fibers, except at the tips of muscle fibers close to the myotendinous junction (Chiquet and Fambrough, 1984; Koch et al., 1995). Moreover, endo- and perimysial fibroblasts normally express high amounts of tenascin-Y, an ECM protein with similar structure and function to tenascin-C, but with a more widespread and often complementary expression pattern compared to tenascin-C (Hagios et al., 1996, 1999). Avian tenascin-Y has the same tissue distribution as the closely related mammalian tenascin-X, of which a mutation in humans is associated with a type of Ehlers-Danlos syndrome (Burch et al., 1997). Thus, skeletal muscle also allowed us to study the possibility that two members of the same ECM gene family could be differentially regulated by mechanical stress.

Overload of chick slow tonic anterior latissimus dorsi (ALD) muscle is a well established in vivo model in which mechanical stress on skeletal muscle can be applied intermittently. After a few days, chronic loading of ALD has been reported to induce a massive rate of muscle growth due to enlargement (hypertrophy) and new formation (hyperplasia) of muscle fibers (Sola et al., 1973). An important role for changes in gene expression in this model is indicated by the fact that, with time, the total DNA, RNA and protein levels are significantly elevated upon loading.

Previous studies characterizing the set of genes induced with overloading of ALD focused on muscle-specific genes. For example, transcription of slow-myosin isoform *SM-2* and transcription factors *myogenin* and *Myf5* was found to be increased in nascent myofibers in chick ALD within days of loading (Kennedy et al., 1988; Lowe et al., 1998).

Limited information exists on changes in gene expression in the connective tissue following stretch of ALD. Early reports demonstrated that synthesis and volume density of bulk collagen and noncontractile tissue are increased in chronically loaded ALD muscle (Alway et al., 1989; Laurent et al., 1978), indicating that transcription of ECM proteins might be activated. However, no studies on the time course of induction of specific ECM proteins have been done so far with this model. Our data presented here demonstrate that upon loading of skeletal muscle, different spatial and temporal patterns of regulation are observed for various ECM proteins such as tenascin-C, tenascin-Y and collagen XII. While some of the responses are relatively slow, others are rapid enough to be compatible with a direct action of tensile stress on the expression of specific ECM genes in muscle connective tissue cells.

MATERIALS AND METHODS

Stretch of skeletal muscle

The State Animal Protection Commission (Amt für Landwirtschaft des Kantons Bern, Switzerland) has approved all protocols used in these experiments. Young chickens (White Leghorn, Wüthrich Farm, Belp, Switzerland) were received at 7–11 weeks of age. They were housed in the animal care facilities at the Ethological Station of the University of Bern and received chicken chow and water ad libitum. The left anterior latissimus dorsi (ALD) muscle of chicken was loaded by fixing a weight corresponding to 10% of the animal's body mass on the left wing with elastic tape. After 4, 10, 24, 36 and 168 hours of the experiment 4-6 animals per time point were anesthetized. For an additional 5 animals the wing was loaded for 36 hours and then released from loading for 24 and 120 hours by removing the weight. Loaded and contralateral control ALD were removed surgically, weighed and frozen in nitrogen-isopentane as previously described by Flück et al. (1999). After surgery, animals were killed by cervical dislocation. Muscles were stored at −70°C in sealed tubes until use. The wet mass of the loaded muscle relative to the contralateral control increased by 44±17%, 42 ±7%, 92 ±13%, 78±12% and 110±9% after 4, 10, 24, 36 and 168 hours of loading, respectively. Removal of the weight after 36 hours initiated regression of wet muscle mass compared to muscles that were only subjected to 36 hours of loading.

Protein extraction and immunoblotting

20 μm cryosections from the belly portion of the ALD muscle were prepared. 60 sections from one muscle were pooled in a frozen Eppendorf tube and extracellular matrix proteins were extracted in 200 μl deoxycholate buffer (50 mM Tris-HCl pH 7.8, 2% deoxycholate, 2 mM EDTA, 5 mM N-ethyl maleimide, 2 mM phenylmethylsulfonyl fluoride) with cycles of vortexing, pipetting and incubation on ice (Trächslin et al., 1999). Soluble protein was recovered in the supernatant fraction after centrifugation (4 minutes, 5000 ***g*** at 4°C). Protein concentration was determined (BCA assay; Sigma, Buchs, Switzerland), and samples were denatured by diluting to 1 μg/μl protein in SDS-PAGE loading buffer containing DTT. 20 μg protein samples were run on 5% SDS-PAGE gels, which were then processed for immunoblotting as described previously (Flück et al., 1999). Blots were stained with Ponceau S to verify equal loading and transfer. The membrane was rinsed in H_2O and TTBS (20 mM Tris base, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and blocked for 1 hour at room temperature in blocking solution (2.5% nonfat dry milk, 1% BSA in TTBS). The membrane was then incubated for 1 hour at room temperature with rabbit antibodies (1:1000 dilution in blocking solution) specific for chicken tenascin-C (#474; Chiquet and Fambrough, 1984), tenascin-Y (#682; Hagios et al., 1996) and collagen XII (#522; Koch et al., 1995), respectively, washed with TTBS, and incubated for 1 hour at room temperature with a 1:5000 dilution in blocking solution of peroxidase-conjugated goat anti-rabbit IgG (ICN Biomedicals GMBH, Germany). The membrane was then washed with TTBS. The signal was detected by enhanced chemoluminescence (SuperSignal®West Pico from Pierce; Socochim SA, Switzerland), recorded on Kodak XAR5 film and imported into Adobe Photoshop®5.0 by using an AGFA Studiostar scanner that was operated by a Power Macintosh G3.

Immunohistochemistry

Cryosections (12 μm thick) were prepared, mounted on glass slides (SuperFrost®Plus; Menzel-Gläser, Germany), air dried and stored (for 0.5-5 days) at –20°C. Upon use, sections were fixed in cold acetone and wetted in PBS. Tissue peroxidase activity was quenched (10 minutes, 0.6% H_2O_2 in methanol), then sections were washed in PBS and blocked with 10% FCS in PBS. Then sections were

incubated (all incubations were for 1 hour at room temperature) with antibodies specific for chicken ECM proteins (see above). After washing in PBS, the sections were reacted with peroxidase-conjugated goat anti-rabbit IgG (1:2000; ICN Biomedicals GMBH, Germany), again washed with PBS and immunoreactivity was detected with 3-amino-9-ethylcarbazole substrate (Sigma, Buchs, Switzerland). The color reaction was stopped with water, nuclei were counterstained with Hematoxylin, and the sections embedded in Aquamount (BDH Laboratory Supplies, Poole, England). The stain was visualized on slide film (Ektachrom 64T, Kodak) by use of a microscope-photograph system (Vanox-S, Olympus). Slides were scanned using a Nikon SF-200 slide-scanner operated by a Power Macintosh G3 using the Nikon Scan 2.0 interface, and imported in JPEG format into Adobe Photoshop®5.0.

For detection of chicken macrophages, sections were quenched and blocked, reacted with a 1:5 dilution of monoclonal antibody 51-2 (Kornfeld et al., 1983), then with goat anti-mouse whole IgG (1:500; ICN Biomedicals GMBH, Germany) followed by mouse-anti-peroxidase complex (1:5000; Jackson Laboratories, West Grove, PA, USA) with intermittent washing steps. Immunoreactivity was visualized essentially as described above.

Northern blotting

Total mRNA was isolated from frozen ALD muscle using the RNeasy midi-kit (Qiagen AG, Basel, Switzerland), separated on 1% agarose gels containing formaldehyde, and blotted onto nylon membranes (Gene Screen; NEN Life Science Product Inc., Boston, MA, USA). Blots were performed with radiolabeled *tenascin-C* or *tenascin-Y* cDNA probes and washed as described previously (Hagios et al., 1996; Trächslin et al., 1999). Stripping of the membrane was performed according to the manufacturer's instructions. Signal was recorded on XAR-5 film (Kodak).

In situ hybridization

Digoxygenin (DIG)-labeled sense and antisense RNA probes specific for chick tenascin-C and tenascin-Y mRNA were synthesized from linearized bluescript plasmids containing the respective cDNAs (Spring et al., 1989; Hagios et al., 1996) with the DIG High prime labeling and detection Starter kit I (Roche Diagnostics Ltd., Rotkreuz, Switzerland), and hydrolyzed to a size of 300 bp prior to hybridization.

Cryosections were prepared as described above and stored overnight at –70°C. After fixation (4% paraformaldehyde in PBS, 20 minutes) followed by washes in PBS, the sections were digested with 5 μg/ml proteinase K (Roche Diagnostics Ltd., Rotkreuz, Switzerland) in 40 mM EDTA, 0.1 M Tris-HCl, pH 8, for 20 minutes. Digestion was stopped with 2 mg/ml glycine in PBS for 1 minute, and slides were fixed again with 4% paraformaldehyde in PBS for 20 minutes. Sections were acetylated for 5 minutes in 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8. The slides were prehybridized with 350 μl hybridization buffer (50% formamide, 4× SSC, 2× Denhardt's, 2% dextran sulfate and 1 mg/ml yeast tRNA) in a moist chamber for 5 hours. Then 40 μl hybridization buffer containing heat-denatured (3 minutes at 80°C) DIG-labeled RNA probes (diluted 1:5-1:20) were applied per slide, covered with a coverslip, sealed and incubated for 16 hours at 60°C. Slides were washed in 0.2× SSC at 60°C for 30 minutes followed by digestion with RNAse A (10 μg/ml) in 2× SSC for 5 minutes at 37°C. Slides were submerged in 2× SSC containing 50% formamide for 30 minutes and then equilibrated in 0.2× SSC at 20°C. For detection of DIG-labeled RNA, the DIG High prime labeling and detection Starter kit I (Roche Diagnostics Ltd., Rotkreuz, Switzerland) was used. After development, sections were washed in water and mounted in Kaiser's glycerol gelatine (Merck AG, Switzerland). Finally the staining pattern was recorded and processed as described before under immunohistochemistry.

RESULTS

Loading of chicken ALD skeletal muscle induces de novo expression of tenascin-C but not tenascin-Y protein

In order to analyze expression of tenascin-C and tenascin-Y protein in chicken skeletal muscle as a function of loading, we made use of previously characterized antisera against the chicken homologues (Chiquet and Fambrough, 1984; Hagios et

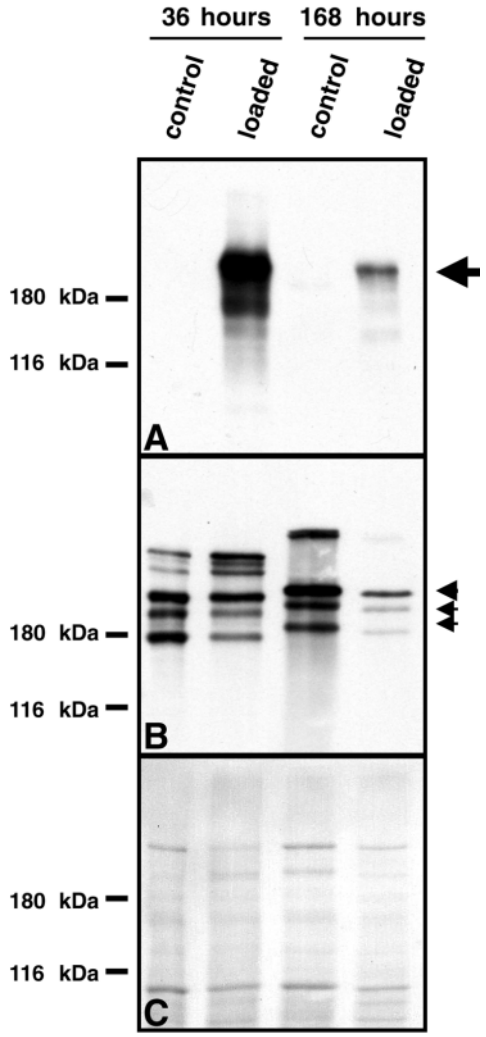

Fig. 1. Expression of tenascin-family proteins in loaded skeletal muscle. Chicken ALD skeletal muscles were loaded for 36 and 168 hours, respectively, loaded and contralateral control ALD were harvested and protein extracts prepared as described in Materials and Methods. Equal amounts of protein (20 μg) were separated on 5% SDS-PAGE gels, subjected to immunoblotting analysis of tenascin-C and tenascin-Y protein using ECL and the signal recorded on film. Films show immunodetected tenascin-C (A) and tenascin-Y (B) protein in extracts from control and loaded ALD from the same animals. (C) Ponceau S-stained membrane showing that equal protein amounts were loaded. The positions of tenascin-C and tenascin-Y variants, respectively, are indicated by arrows; molecular mass markers are indicated to the left. Representative results from 3 animals are shown. Note the massive induction of tenascin-C protein after loading but the reduced tenascin-Y protein when loading was maintained for longer.

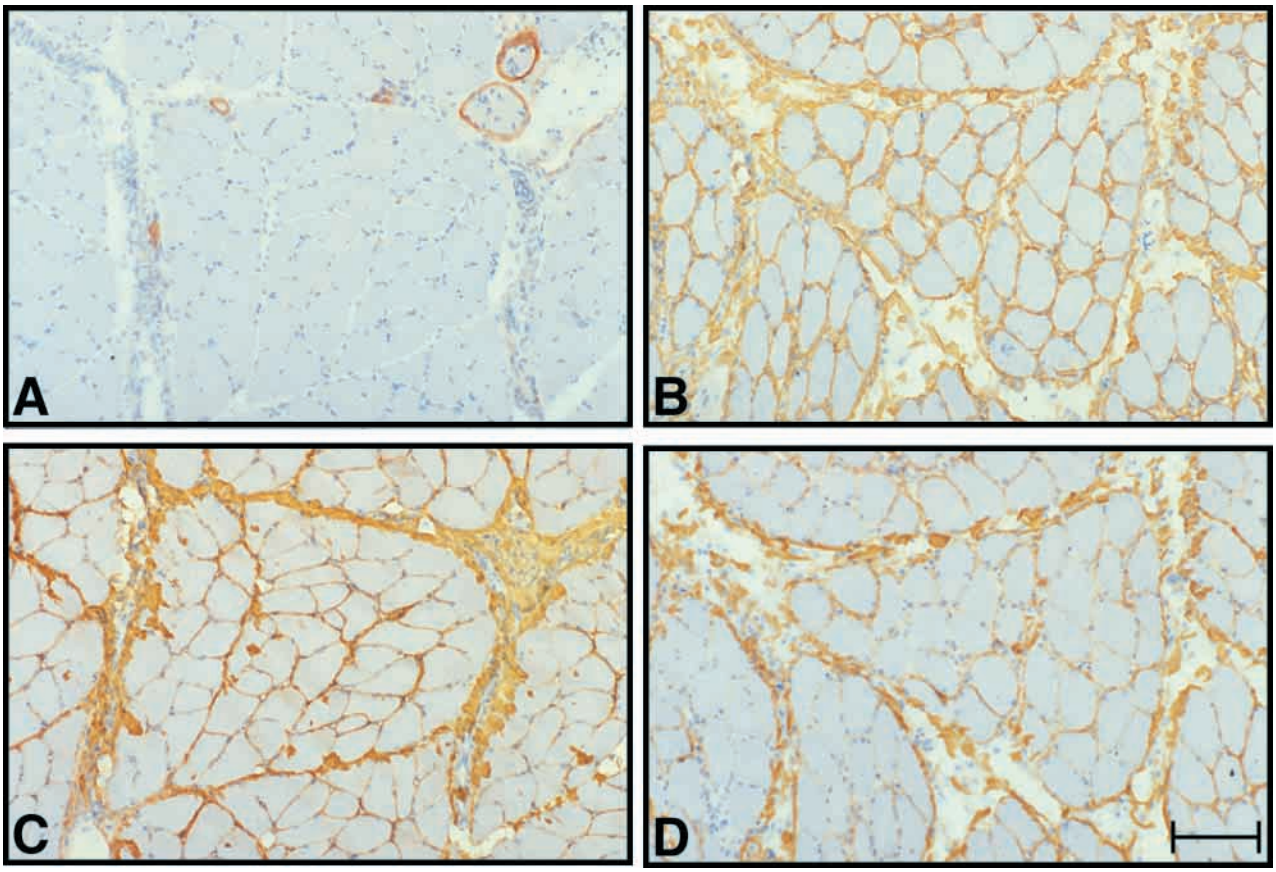

Fig. 2. Localization of tenascin-family proteins in loaded skeletal muscle. Consecutive cryosections (12 μm) from 36-hour loaded (B,D) and respective contralateral control ALD muscle (A,C) were subjected to immunohistochemical analysis for tenascin-C (A,B) and tenascin-Y (C,D). Immunostaining appears in brown-yellow. Nuclei were stained with Hematoxylin and are blue. The part of the sections corresponding to the same area of the muscles is shown. Bar, 100 μm.

al., 1996). We first confirmed that tenascin-C (190-230 kDa) and tenascin-Y (170 kDa, 220 kDa doublet, 300 kDa, 400 kDa doublet) splice variants were present in extracts from the myotendinous junction and from the middle portion of control ALD muscle, respectively (not shown). A very faint level of tenascin-C expression was seen in extracts from the middle portion (Fig. 1A). In published reports, the earliest expressional responses in avian ALD muscle were observed after 24-48 hours of loading with 10% of the body mass (Lowe et al., 1998; Mitchell et al., 1999). Based upon these observations, we chose a similar amount and duration of loading in initial experiments to assess the eventual changes in tenascin-C and tenascin-Y protein expression. Immunoblots of extracts prepared from the middle portion of ALD revealed that 36 hours of loading strongly induced de novo expression of 190-230 kDa tenascin-C variants (Fig. 1A). In contrast, the total amount of tenascin-Y variants was barely affected with 36 hours of loading (Fig. 1B).

When loading was maintained for 168 hours, tenascin-C protein expression stayed elevated, however at a lower level, while the amount of tenascin-Y isoforms was moderately reduced (Fig. 1A,B).

Ectopic expression of tenascin-C in endomysium of loaded ALD skeletal muscle

We performed immunohistochemical analysis on cryosections from the middle portion of ALD to identify the location of de novo tenascin-C expression in loaded ALD muscle. Tenascin-C expression in 36-hour loaded ALD muscle was found to be most prominent in endomysium, whereas in control ALD it was principally confined to blood vessels and perineurium (Fig. 2A,B). Tenascin-Y expression in 36-hour loaded ALD muscle appeared reduced in the endomysium relative to control ALD (Fig. 2C,D).

Early and reciprocal changes in tenascin-C and tenascin-Y transcripts in loaded skeletal muscle

De novo synthesis of any protein is usually preceded by an increase in the corresponding mRNA. We determined the time course and cellular origin of presumed tenascin-C mRNA induction. Northern blot experiments with ALD muscles subjected to loading of short duration showed that tenascin-C mRNA was induced de novo in the middle portion of the muscle after only 4 hours of loading and increased only slight more with loading of longer duration (Fig. 3A). Reciprocally, the level of tenascin-Y transcripts was reduced relative to the control after 4 hours of loading (Fig. 3B). In situ hybridization experiments revealed that after 4 hours of loading, expression of mRNA coding for tenascin-C was strongly induced in a uniform manner in endomysial fibroblasts while it was only faintly detectable in control ALD (Fig. 4A-D). In contrast,

expression of tenascin-Y mRNA in fibroblasts of ALD muscle appeared already reduced after 4 hours of loading (Fig. 4E,F). When loading was maintained for 24 hours, tenascin-Y mRNA disappeared while tenascin-C mRNA levels were still increasing in the endomysium (not shown).

Induction of tenascin-C protein correlates with application of load but not with macrophage infiltration

It has been reported that infiltration of dystrophic or regenerating muscle by inflammatory cells correlates with increased deposition of extracellular matrix proteins (Gullberg et al., 1997; Gullberg, 1998). A minor degree of injury has indeed been observed in avian ALD muscle overloaded for up to 3 days (Winchester and Gonyea, 1992). We analyzed the possibility that inflammatory responses could occur rapidly after onset of loading. Using an established antibody against chicken macrophages (Kornfeld et al., 1983), which are the first immune cells to infiltrate damaged tissue, we observed scattered macrophages in control ALD but found no signs of increased macrophage infiltration in ALD muscle loaded for 4 hours (Fig. 5A,B). Patches of macrophage infiltration were observed after 24-36 hours of loading (Fig. 5C). The uniform pattern of de novo synthesized *tenascin-C* mRNA in the endomysium after 4 hours of loading therefore did not correlate with the inflammation responses.

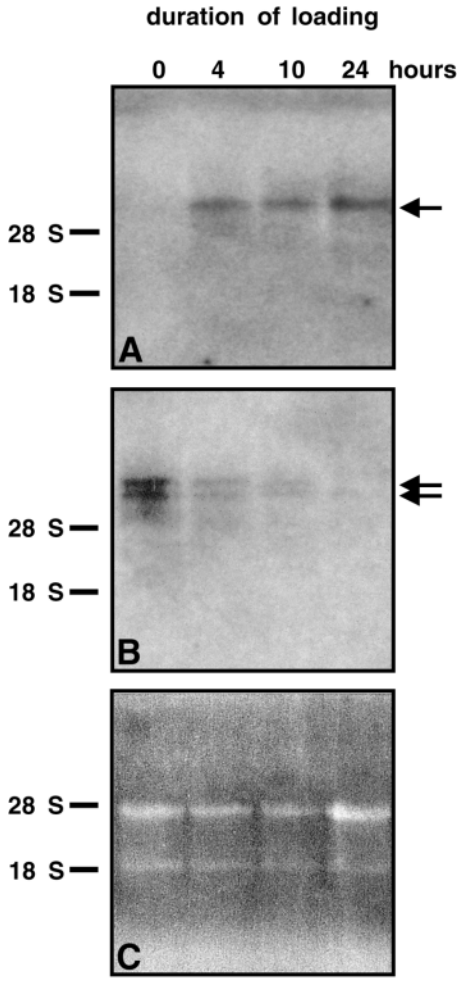

Fig. 3. Early loading-induced changes in tenascin-C and tenascin-Y transcripts. Total RNA from ALD muscles subjected to 0, 4, 10 and 24 hours of loading was separated and northern blotted, and tenascin-C and tenascin-Y mRNA species were detected on the same membrane which was stripped in between as described in Materials and Methods. The detected signals for (A) tenascin-C and (B) tenascin-Y mRNA, as well as the loading control (28S and 18S RNA) visualizing the ethidium bromide stain of the same membrane (C). The positions of tenascin-C and tenascin-Y transcripts, respectively, are indicated by arrows. The locations of 18S and 28S RNA species are indicated to the left. Note the strong induction of tenascin-C and reduction of tenascin-Y transcripts within 4 hours of loading.

Mechanical loading of skeletal muscle controls expression of collagen XII differently from tenascin-C

It was possible that early induction of tenascin-C on loading of skeletal muscle reflects a general rapid activation of ECM protein expression and that the decline in tenascin-Y mRNA in response to loading is an exception rather than the rule. We therefore analyzed expression of tenascin-C protein relative to the ECM protein collagen XII, which is distinct from the tenascin family. Confirming the mRNA data, tenascin-C protein variants were induced as early as after 4 hours of loading (Fig. 6A). Small (220 kDa) and large (320 kDa) splice variants of collagen XII were induced above the level seen in control ALD muscle after 10 hours of loading but to a lower extent than for tenascin-C (Fig. 6B). Immunohistochemical and in situ hybridization analysis demonstrated that increased collagen XII mRNA and protein expression were confined to endomysium of ALD skeletal muscle (not shown). Additionally we found that expression of fibronectin mRNA and protein and collagen I transcripts was induced in endomysium of loaded skeletal muscle, but again with apparently slower kinetics and magnitude than for tenascin-C (not shown).

Finally we asked whether a regression of tenascin-C and collagen XII expression occurs in chicken ALD muscle when the load is removed from the wing after 36 hours. It was found that the tenascin-C protein level observed in ALD after 36 hours of loading was significantly reduced 48 hours after removal of the load and had nearly disappeared 120 hours later (Fig. 6C). The level of tenascin-Y protein seemed not to be affected by the same treatment (not shown). Release of muscle from 36 hours of loading also caused a reduction of the collagen XII protein level, but this decrease was not fully reversible and became significant only after 120 hours (Fig. 6D). Enhanced fibronectin protein did not significantly decrease within 120 hours of release from loading (not shown).

DISCUSSION

Mechanical stimulation is essential for the homeostasis of connective tissues (Wolff, 1892; Bloomfield, 1997; Johnson, 1998). However, the sequence of molecular events underlying adaptation of the extracellular matrix to mechanical stress is only partially known (Lambert et al., 1992; Langholz et al., 1995). Tenascin-C and collagen XII are two ECM proteins found together in tissues bearing high tensile stress, and in cultured cells the synthesis of both proteins is induced by

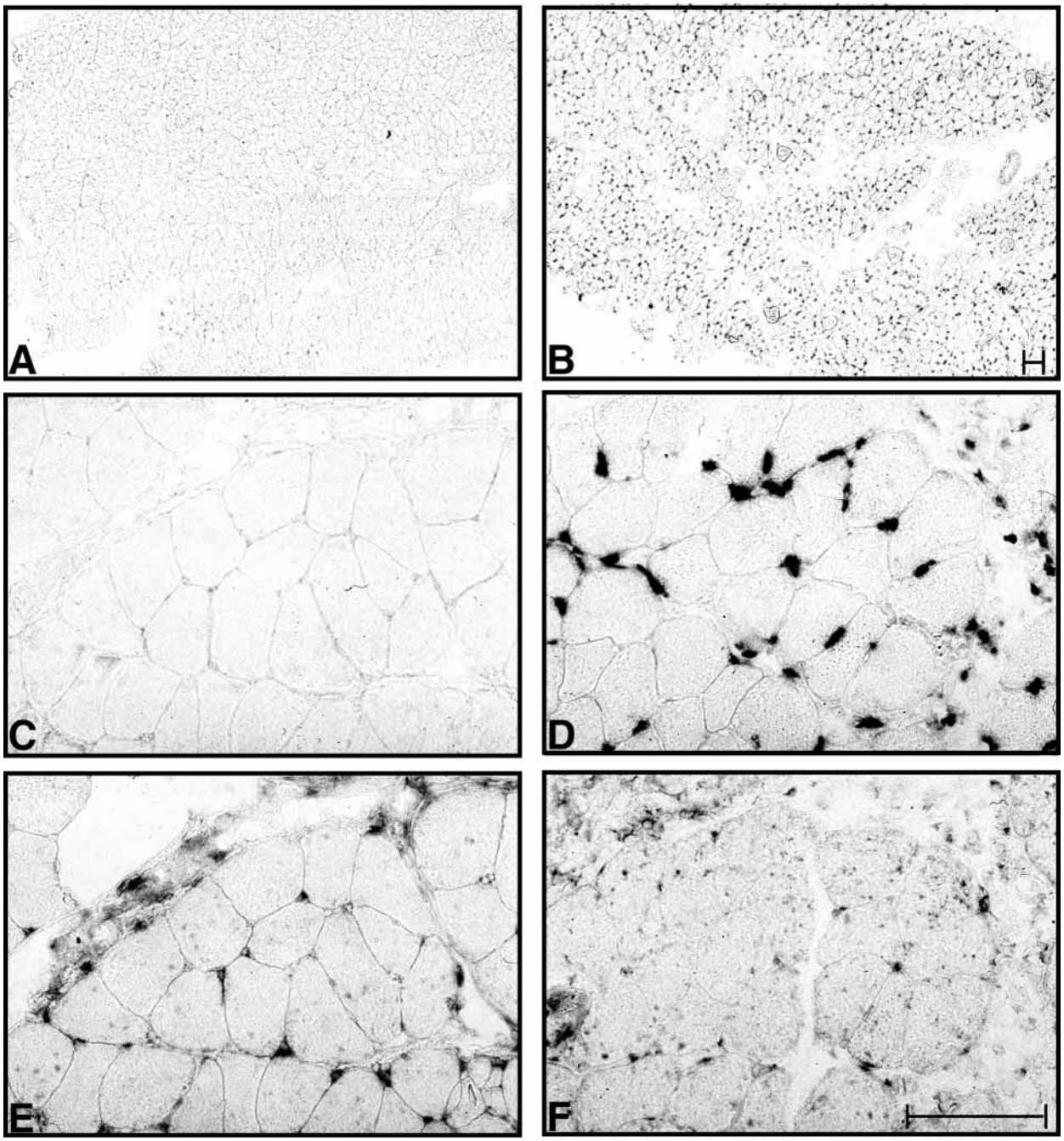

Fig. 4. Localization of tenascin-family transcripts. Cryosections from 4-hour loaded (B,D,F) and control (A,C,E) ALD from the same animal were subjected to in situ hybridization with DIG-labeled RNA probes coding for tenascin-C (A-D) and tenascin-Y (E,F) antisense RNA. (A,B) 40-fold, (C-F) 280-fold magnification. Positive signals appear dark gray. Hybridization with sense probes did not detect any distinct staining on these sections. Bars, 100 μm.

mechanical stimulation (Chiquet-Ehrismann et al., 1994; Trächslin et al., 1999). Several reports indicate that expression of tenascin-C and collagen XII is in part controlled by tensile stress in living tissues. Collagen XII is increased in the periodontal ligament upon exprimental tooth movement (Karimbux and Nishimura, 1995). Tenascin-C is induced in the arterial walls of rats in a hypertension model (Mackie et al., 1992) and in the periosteum of rat ulnae after applying an external load (Webb et al., 1997). Recently, Järvinen et al. (1999) showed that the normal level of tenascin-C protein in the osteotendinous junction of the rat quadriceps muscle was markedly reduced 3 weeks after cast immobilization of the leg, and was partially restored within a week of remobilization. This finding was taken as 'conclusive evidence' that tenascin-C is regulated by mechanical stress in vivo.

However, in this as well in the other studies mentioned, changes in tenascin-C or collagen XII expression were observed in vivo only after prolonged periods of time, and they

were not compared to those of other ECM proteins. Thus, the observed effects might have been a secondary and rather general consequence of much earlier events triggered by changes in mechanical load, such as the release of growth factors by cells of the stimulated tissue (Sadoshima and Izumo, 1997) or by invading inflammatory cells (Gullberg, 1998). In fact, it is well known that *tenascin-C* mRNA and protein levels are regulated by growth factors and hormones, eg. by TGF-β (Pearson et al., 1988), angiotensin II (Mackie et al., 1992) and glucocorticoids (Ekblom et al., 1993). In addition, enhanced tenascin-C expression has been correlated with an inflammatory response in various myopathies (Gullberg et al., 1997). Thus, it is important to study the time course of changes in gene expression in response to mechanical stress in vivo in order to distinguish primary from secondary events.

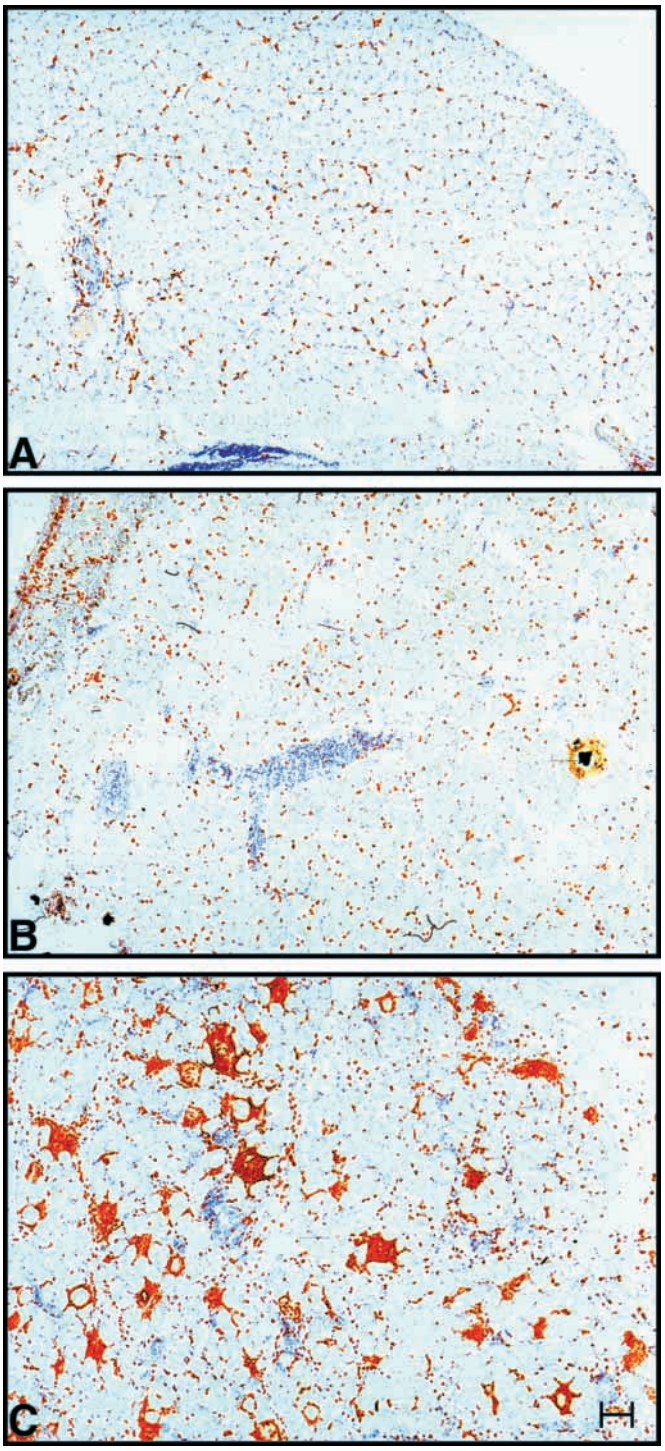

Fig. 5. Macrophage infiltration in loaded skeletal muscle. Consecutive cryosections (12 μm) from control (A), 4-hour (B), and 24-hour (C) loaded ALD muscle were subjected to immunohistochemical analysis for chicken macrophages as described in Materials and Methods. Immunostaining appears in orange and Hematoxylin-stained nuclei are blue. Bar, 100 μm.

The data presented here provide compelling evidence that a changed ECM production in skeletal muscle connective tissue is not simply part of a generalized systemic effect of mechanical loading. Instead, different spatial and temporal patterns of regulation are observed for specific ECM proteins such as tenascin-C, tenascin-Y and collagen XII. Particularly striking is the ectopic induction of tenascin-C mRNA and protein in the endomysium of ALD muscle within only 4 hours of loading, concomitant with a downregulation of tenascin-Y mRNA. These two responses precede an increase in macrophages, which can only be observed hours later. The time course of immigration of immune cells has been investigated after acute muscle damage (bupivacaine-induced myonecrosis). In this model macrophages and lymphocytes were found to infiltrate damaged muscle after 24 hours after treatment (Orimo et al., 1991). A small number of polymorphonuclear lykocytes (PMNs) were observed within 30 minutes of treatment; however, their distribution was mainly intra- and perivascular and remained patchy even after 12 hours. If tenascin-C expression was induced by soluble factors released by PMNs in our case, one would expect a patchy perivascular expression 4 hours after applying the load. Therefore, although it is not possible to definitely rule out the involvement of soluble mediators in an in vivo model, the rapid and homogeneous induction of tenascin-C mRNA points to a direct action of mechanical stress on gene activation in endomysial fibroblasts. In contrast, the slower time course and only partial reversibility of induction observed for collagen XII (as well as for fibronectin and collagen I) is in agreement with a secondary (e.g. regenerative) response of the muscle connective tissue to excess mechanical load.

The level of tenascin-C protein correlates well with the expression of the corresponding mRNA after 0-36 hours of loading (Figs 1-3, 6). In contrast, the expression of tenascin-Y protein variants as detected by immunoblotting was barely affected until 36 hours of loading and did not correlate with the significant reduction of the corresponding mRNAs (Figs 1-3). This discrepancy can be explained by the fact that many extracellular matrix proteins exhibit slow turnover rates. Although such a rate has not been measured for tenascin-Y so far, it is to be expected that the protein present at the start of the experiment persists for days, even if its mRNA level drops rapidly after applying the load.

What could be the mechanism by which stretching of the muscle so rapidly changes the mRNA levels of the two tenascins in endomysial fibroblasts? Loading the wing rapidly stretches the adductor muscle ALD through a gravitational pull (Alway et al., 1989). The resulting maximal increase in length is 30% and is achieved within the first 24 hours of loading (Winchester et al., 1991). This lengthening

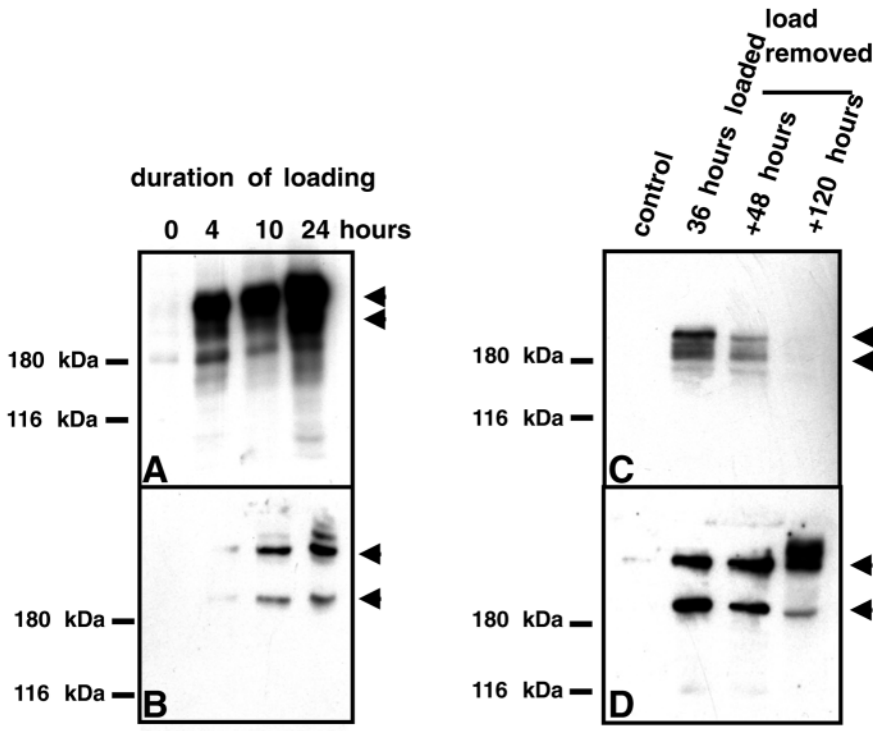

Fig. 6. Load-induced expression of tenascin-C in skeletal muscle is different from that of collagen XII. Equal amounts of protein (10 μg) in extracts prepared from control and loaded (for 4, 10 and 24 hours) ALD were separated on 5% SDS-PAGE gels and subjected to immunoblotting analysis for tenascin-C (A) and collagen XII (B). Alternatively, extracts were prepared from contralateral control ALD, 36-hour loaded ALD and 36-hour loaded ALD muscle 48 or 120 hours after removal of the load, and an equal amount of protein (10 μg) was subjected to immunoblotting analysis for tenascin-C (C) and collagen XII (D). Tenascin-C and collagen XII protein isoforms, respectively, are indicated by arrowheads. Molecular mass markers are indicated to the left. Note that tenascin-C protein induction is more rapid than that of collagen XII and is completely reversible.

produces strain that is not only transmitted in series through sarcomeres, but also laterally to the connective tissue, which has an important role in force transmission in skeletal muscle (Hujing, 1999; Monti et al., 1999). Thus, the connective tissue cells of loaded and stretched ALD muscle are likely to be exposed to more passive load than those of the unloaded control. Since tenascin-C induction does not depend on an inflammatory response in our case, tenascin-C expression in muscle weakened by disease (Gullberg et al., 1997) could at least in part be caused by passive chronic overload as well. In vitro, tensile stress induces expression of tenascin-C and collagen XII in fibroblasts cultured on fibrillar collagen matrices (Chiquet-Ehrismann et al., 1994; Trächslin et al., 1999). Published data indicate that regulation occurs at the transcriptional level, presumably via stretch-responsive cis-acting regions in the promoter of the corresponding gene (Chiquet et al., 1998; Chiquet, 1999). A possible signal transduction mechanism has been postulated for tenascin-C induction in cardiac muscle cells cultured on silicon membranes and subjected to cyclic stretching. In this case, reactive oxygen species produced by the mechanically stimulated cells themselves seemed to induce nuclear translocation of the transcription factor NFkB, which transactivated an enhancer region in the tenascin-C gene promoter (Yamamoto et al., 1999). In the in vivo loading experiments presented here, the very rapid and uniform changes in the levels of tenascin-C in endomysial fibroblasts strongly suggest that a similarly direct, cell-autonomous regulatory process is effective, at least for this gene.

The differential adaptation of expression levels of various ECM proteins in response to stretch in vivo implies that the activity of their gene promoters and/or the turnover of their transcripts are governed by different cellular and molecular mechanisms. Thus, although functionally similar stretch-responsive regions have been identified in the promoters of the *tenascin-C* and the *collagen XII* genes by in vitro transfection experiments (Chiquet-Ehrismann et al., 1994; Chiquet et al., 1998), the upstream signaling pathways involved are likely to be different for these two genes since their time course of induction is different after loading in vivo. The promoter of the *tenascin-Y* gene has not yet been analyzed. It will be interesting to study whether the same stretch-triggered signaling pathway that activates *tenascin-C* gene transcription in turn acts on a silencer sequence in the *tenascin-Y* promoter, or whether an entirely different mechanism is responsible for the opposite response observed for this gene.

The reciprocal regulation of tenascin-C and tenascin-Y transcripts upon stretching of skeletal muscle is reminiscent of their complementary expression pattern in many tissues (Hagios et al., 1996, 1999). The functional consequences of such a switch are not yet known. Nevertheless, to our knowledge the expressional changes of the tenascin-family mRNAs are earlier than any other changes reported to occur with loading of skeletal muscle in vivo (Kennedy et al., 1988; Lowe et al., 1998; Mitchell et al., 1999). This implies that adaptation of the levels of these ECM proteins in muscle connective tissue plays an early and important role in the processes that occur during loading-induced skeletal muscle growth and hypertrophy (Jablecki et al., 1976). The experiments reported here set the stage to test such a hypothesis.

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