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Differential expression of tenascin after denervation, damage or paralysis of mouse soleus muscle

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Summary

The expression of the extracellular matrix molecule tenascin was studied by immunocytochemistry and Western blotting in soleus muscles of adult mice after nerve damage (denervation), muscle injury (induced by enforced running or freezing) and functional block of synaptic transmission (botulinum toxin). Enhanced expression of tenascin in the extracellular spaces around focally damaged muscle fibres was found already 10 h after onset of running on a motor-driven treadmill which causes muscle injury in soleus muscle. Tenascin expression reached a peak at 2–3 days post-exercise, after which it declined gradually and became undetectable by two weeks after injury. Similarly, cryo-damage of soleus muscles *in situ* led to upregulation of tenascin. Chronic muscle denervation after sciatic nerve transection caused a persistent (studied up to 31 days) expression of tenascin at denervated endplates and in intramuscular nerve branches but not in other tissue compartments. Local application of botulinum toxin Type A, which results in muscle inactivity but not in tissue degeneration, however, did not induce tenascin expression 12 h to 12 days post-injection. Expression of tenascin after denervation and muscle damage, but its absence after paralysis, were verified by SDS-PAGE and Western blot analysis. Independent of the type of injury (muscle, nerve or both) the known major isoforms of mouse tenascin, as judged by M_r comparison, were re-expressed, with no preponderance of individual M_r forms. These results show that tenascin expression in adult muscles is induced by both axon and muscle fibre damage but not by muscle inactivity. In contrast, NCAM, in accordance with previous observations, showed enhanced expression both as a result of inactivity and in association with tissue repair.

Introduction

The extracellular matrix (ECM) glycoprotein tenascin was first described by Chiquet and Fambrough (1984 a,b) as myotendinous antigen in the chicken. Its mouse equivalent, J1-200/220, was independently discovered, together with two other J1-forms not related to tenascin (J1 160 and 180 kD), as a constituent of the L2/HNK-1 family of neural cell adhesion molecules (Kruse *et al.*, 1985). The homology of J1-200/220 and tenascin has been proven with immunochemical methods (Faissner *et al.*, 1988). Furthermore, it has been shown that in the chicken tenascin and cytotactin (originally described by Grumet *et al.*, 1985) are identical (Pearson *et al.*, 1988; Jones *et al.*, 1989). Other ECM proteins structurally related to tenascin are hexabrachion (Erickson & Inglesias, 1984), GMEM (Bourdon *et al.*, 1985) and polypeptide 150/225 (Gulcher *et al.*, 1986). As revealed by electron microscopy, tenascin and related molecules typically have a 'hexabrachion' structure, i.e. they consist of six arms connected to a central globule (Erickson & Inglesias, 1984; Spring *et al.*, 1989; Lochter *et al.*, 1991). Each arm has a cystein-rich domain contributing to the central globule, followed by a thin part of 13.5 epidermal growth factor (EGF) – like repeats and a thicker portion built up by a variable (due to alternative splicing) number of fibronectin Type III repeats; the arm terminates in a globular carboxyterminal domain with fibrinogen homology (Jones *et al.*, 1989; Spring *et al.*, 1989; Siri *et al.*, 1991; Weller *et al.*, 1991).

Tenascin is detectable in various organs during specific stages of development but its expression in adult tissues is sparse (for reviews see Chiquet, 1989; Erickson & Bourdon, 1989). However, re-expression of tenascin in adults has been observed after injury of

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the peripheral nerve (Sanes *et al.*, 1986; Gatchalian *et al.*, 1989; Martini *et al.*, 1990; see also Daniloff *et al.*, 1989 for cytotactin) and the skin (Mackie *et al.*, 1988). Several cell types, e.g. astrocytes, Schwann cells and fibroblasts, have been shown to synthesize tenascin (Kruse *et al.*, 1985; Chiquet-Ehrismann *et al.*, 1986; Faissner *et al.*, 1988; Gatchalian *et al.*, 1989; Martini *et al.*, 1990). Low level of cytotactin has also been observed in cultured myoblasts (Daniloff *et al.*, 1989).

In vitro experiments have shown that tenascin is a repulsive substrate for fibroblasts, tumour cells of epithelial origin and CNS neurons (Chiquet-Ehrismann *et al.*, 1986, 1988; Faissner & Kruse, 1990). On the other hand, tenascin may also promote cell attachment (Kruse *et al.*, 1985; Spring *et al.*, 1989). Tenascin inhibits neurite outgrowth in culture when applied in a soluble form but promotes this outgrowth when present in a substrate-bound form (Lochter *et al.*, 1991). Thus, tenascin appears to have different functions each of which is mediated by a distinct domain of the molecule (Spring *et al.*, 1989; Lochter *et al.*, 1991; Prieto *et al.*, 1992).

From studies on tissue distributions of cytotactin and tenascin during development it has been inferred that they play important roles in cell segregation, migration and aggregation (Edelman, 1988; Chiquet, 1989; Erickson & Bourdon, 1989). On the other hand, from studies on mutant mice lacking tenascin it appears that apparently normal development occurs in the absence of this protein (Saga *et al.*, 1992). Functional roles for these molecules in muscle reinnervation in adult animals have been recently demonstrated in *in vivo* experiments. Mège and colleagues (1992) found that antibodies to cytotactin delay the reinnervation of original synaptic sites on basal laminae in damaged and irradiated frog muscles. In the adult mouse, application of specific antibodies to tenascin onto denervated muscles significantly delays muscle reinnervation as revealed by *in vivo* observations of endplates (Wernig *et al.*, 1992).

In this study we compared, with immunocytochemical methods and Western blotting, the expression of tenascin in normal, denervated, damaged and paralysed muscles in order to discriminate factor(s) associated with tissue degeneration, cell proliferation or muscle inactivity which induce re-expression in the adult neuromuscular system. In addition, comparisons with NCAM, which shows enhanced expression as a result of either inactivity or injury of muscle (Covault & Sanes, 1985, 1986; Daniloff *et al.*, 1986; Moore & Walsh, 1986; Cashman *et al.*, 1987), were made. The results indicate that only local factors associated with tissue damage induce enhanced tenascin expression and that independent of a type of damage (of muscle fibres, in the intramuscular nerve or both) the major forms of tenascin are re-expressed in a similar ratio.

Materials and methods

Animals and experimental procedures

Inbred CBA/J mice of both sexes bred in the laboratory were used at approximately three months of age. The animals were kept under standard laboratory conditions with free access to food and water. All experiments were performed according to the German law for protection of animals.

Muscle denervation ($n = 11$) was performed unilaterally under pentobarbitone anaesthesia (Nembutal, 60 mg kg^{-1}) by transecting and removing part of the sciatic nerve in the thigh (approximately 1 cm). To prevent reinnervation, the end of the proximal stump was sutured to nearby muscles. Muscle paralysis ($n = 12$) was achieved by injection of Type A botulinum toxin (25 pg g^{-1} body weight in phosphate-buffered saline, PBS) into the right calf muscles under anaesthesia. One day after toxin application paralysis of the injected limb muscles was apparent in all animals. Combined cholinesterase-antineurofilament staining in longitudinal sections from two soleus muscles which were studied two and three weeks after injection revealed massive axonal sprouting throughout the whole muscles (comparable to other observations, e.g. Duchen, 1970). A third group of animals ($n = 15$) was exercised on a motor-driven treadmill for a total period of 9 h (3 × 3 h with 30 min rest between the bouts, 14 m min^{-1}, inclination 6°) as described previously (Wernig *et al.*, 1991a). In eight other animals the right soleus muscle was frozen *in situ* with a metal rod precooled in liquid nitrogen under pentobarbitone anaesthesia after which the wounds were closed (Wernig *et al.*, 1991b); cryo-damaged muscles were studied 2–7 days later. Intact animals ($n = 6$) served as controls.

Histological procedures

Soleus muscles were isolated, mounted on liver pieces (Irintchev *et al.*, 1990) and frozen in melting isopentane. With the exception of a few muscles which were sectioned longitudinally, serial cross-sections were cut at 10 μm in a cryostat and collected on gelatin-coated slides. Sections were taken from two defined parts of the muscles: proximal, at the beginning of the intracapsular region of the most proximal spindle (non-endplate region of the muscle), and from the synaptic region in the middle of the muscle (see Wernig *et al.*, 1991a). Sections from each series were stained with Toluidine Blue and for activities of several enzymes (succinate dehydrogenase, myofibrillar ATPase, acid phosphatase, glycogen phosphorylase) to assess signs of muscle damage as described previously (Irintchev & Wernig, 1987; Wernig *et al.*, 1990, 1991a). Degeneration, necrosis and phagocytosis, regeneration of muscle fibres, proliferation of satellite cells, interstitial cellular infiltration are phenomena observed in the acute phase of damage and repair, chronic signs of previous damage are 'split' fibres and centralized muscle nuclei (see Wernig *et al.*, 1990, 1991a).

Antibodies

Rat monoclonal antibodies J1/tn1 (576), J1/tn2 (578) and polyclonal rabbit antibodies to postnatal mouse brain tenascin (pJ1/tn), described previously (Faissner & Kruse, 1990), as well as rat monoclonal anti-NCAM antibody H-28 (Gennarini *et al.*, 1986) were used. Affinity-purified biotin-

conjugated goat anti-rat IgG, normal goat IgG and FITC-streptavidin were used for immunocytochemistry (Jackson Immunoresearch Laboratories, PA).

Immunocytochemistry

Tissue sections were fixed for 3–5 min in methanol at −20° C, air-dried and overlayed with 2% normal goat serum (NGS) in PBS for 30 min at room temperature. Prior to immunostaining sections were typically stained with rhodaminated α-bungarotoxin to visualize the endplates. Incubations with the primary antibodies were made in staining jars containing 30 ml solution which is stable at 4° C for long periods and allows repeated use (Sofroniew & Schrell, 1982). The primary antibodies were diluted 1:1000–1:2000 (tenascin, final concentrations 2.3–3.5 μg ml^{-1}) or 1:100 (NCAM, hybridome supernanant) with PBS containing 0.7% w/v lambda carrageenan (Sigma, nongelling gelatin which reduces background levels) and 0.02% w/v sodium azide as preservative. Incubation was performed overnight at 4° C. Following rinsing in PBS (3 × 15 min, room temperature at this and all later steps), sections were incubated with biotin-conjugated goat anti-rat IgG (1:250)/2% NGS in PBS for 1 h and washed again (3 × 10 min). Finally, slides were overlayed with FITC-streptavidin (1:200 in PBS) for 30 min, washed (3 × 5 min) and embedded in *p*-phenylene-diamine/glycerin. Control incubations, all of which were negative, included omitting of primary or secondary antibody, and substitution of the primary antibody with non-immune rat IgG (5 μg ml^{-1}).

Analysis of muscle tissue by SDS-PAGE and Western blotting

For biochemical analysis, soleus muscles were surgically removed without tendons and immediately shock-frozen in liquid nitrogen. Tissue pieces of comparable size were suspended in 0.4 ml cold extraction buffer consisting of 2.0 M urea, 2 mM EDTA, 2 mM EGTA, 10 mM Tris-HCl, pH 8.5, supplemented with 100 mM ϵ-aminocaproic acid, 1 mM phenylmethylsulphonylfluoride (PMSF), 1.0 μg ml^{-1} α-2-macroglobulin, 5 mM benzamidine-HCl, 1.0 μM leupeptin, 1.0 μM pepstatin and 10 μg ml^{-1} soybean trypsin inhibitor. After three cycles of freezing and thawing the muscle was homogenised with 30 strokes of a glass homogenizer and vigorously vortexed for 30 min at 4° C. Thereafter, the homogenates were centrifuged for 10 min at 10 000 g, supernatants were collected and the pellets were resuspended in an equal volume of extraction buffer. Both supernatants, operationally defined as muscle tissue extracts, and resuspended pellets were subsequently dried in a Speed-Vac vacuum centrifuge. Dried tissue extracts and pellets were dissolved in 150 μl of 2× concentrated SDS-sample buffer, heated for 5 min at 95° C and resolved on 4%–10% SDS-PAGE gradient slab gels. For analysis of samples by Western blotting gels were transferred to nitrocellulose or Immobilon filters which were treated as described previously (Martini *et al.*, 1990). In brief, membranes were blocked for 3 h at room temperature with Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 7.4), containing 4% w/v dried milk powder, 0.1% w/v Tween 20 and 0.05% w/v sodium azide. Subsequently, filters were incubated for 18 h at room temperature with 10 μg ml^{-1} pJ1/tn diluted in TBS containing 4% w/v dried milk powder, 0.1% w/v Tween 20 and 0.05% w/v azide, pH 7.4. Filters were washed 5× in TBS containing 0.1% w/v Tween 20, 0.05% w/v sodium azide and incubated for 2 h at room temperature with ^{125}I-labelled protein A (Amersham, 30 mCi/mg, 150 nCi/ml) in TBS containing 0.1% w/v Tween 20 and 1 mg/ml bovine serum albumin (BSA). After five washes with TBS containing 0.1% w/v Tween 20 the nitrocellulose filters were processed for autoradiography (Faissner *et al.*, 1988, Martini *et al.*, 1990). Extracts and pellets of treated and control muscles were individually investigated. Tissues recovered after distinct experimental schedules and chosen for comparison were resolved on the same gel and tenascin purified from postnatal mouse brain was included as internal reference for molecular weight assignments (Faissner & Kruse, 1990).

Results

Normal muscles

In normal soleus muscles ($n = 5$) tenascin was immunocytochemically undetectable in the endplate (Figs 1 & 2) or in non-endplate regions of the muscle except for the myotendinous junctions. NCAM was present only at neuromuscular junctions and in thin, obviously unmyelinated, axons in intramuscular nerves (see Mirsky *et al.*, 1986; Rieger, 1990).

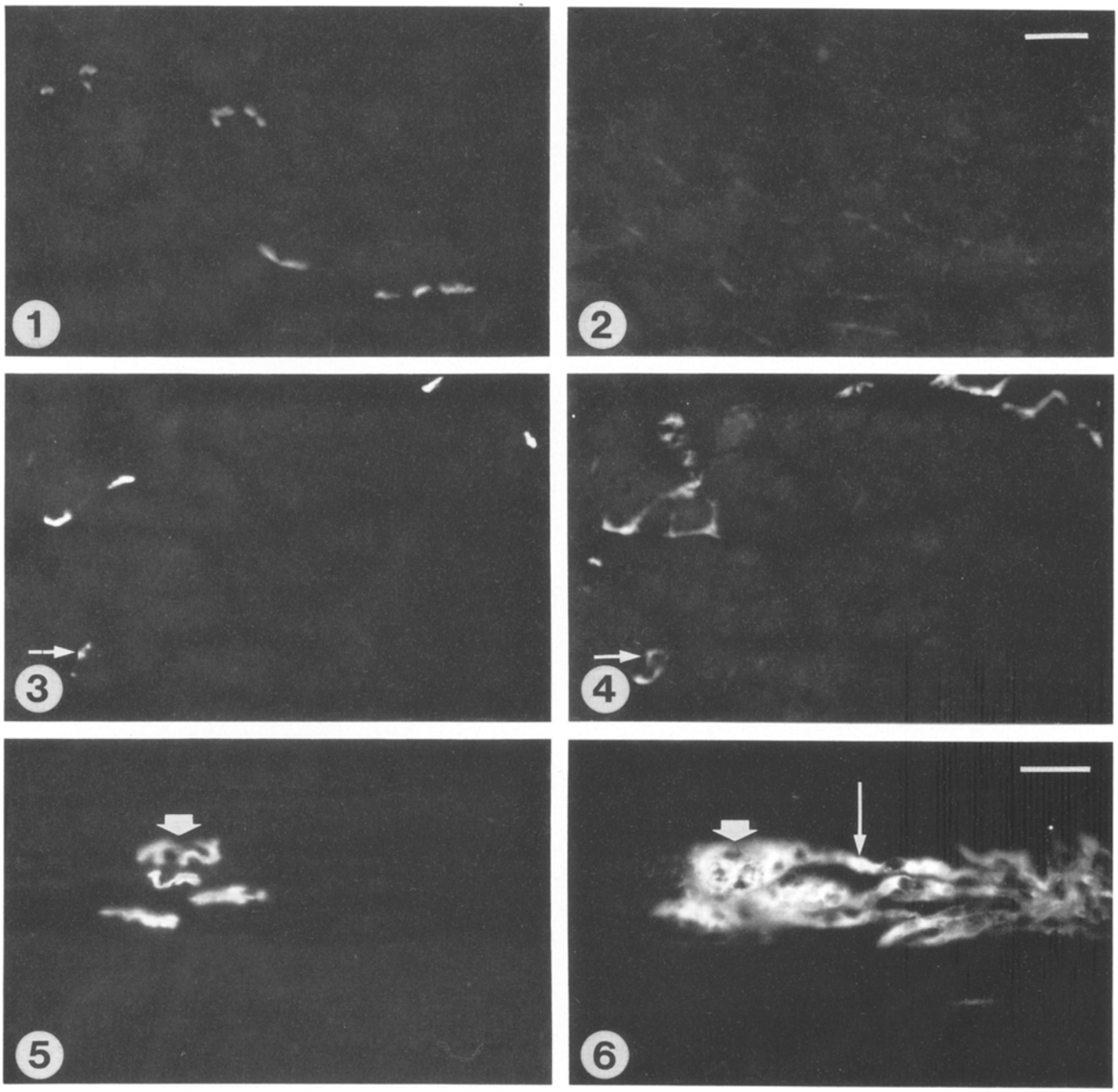
Denervated muscles

Tenascin expression in soleus muscle was seen first 24 h after nerve transection and persisted throughout the period of denervation studied (up to 31 days) (Table 1). Tenascin immunoreactivity was strictly confined, first, to endplates and the interstitial space which closely surrounded endplates (perisynaptic area), and, secondly, to the denervated intramuscular nerve branches (Figs 3–6). Enhancement of tenascin expression followed a distal to proximal course: in endplates and perisynaptic areas it was first seen 24 h after denervation, extended to intramuscular nerve branches at two days, and appeared in the nerve stem at the point of entry into the muscle three days post-denervation. Phagocytic activity, as judged from stainings for acid phosphatase, a lysosomal marker enzyme, increased in the same spatial and temporal sequence observed for tenascin.

In contrast to tenascin, enhanced NCAM expression became detectable as late as three days after nerve transection. NCAM was localized at the surface of muscle fibres and, in numerous but not all fibres, also in the sarcoplasm. Unlike tenascin, reactivity was present all along the length of muscle fibres.

Exercise-induced injury

A single episode of enforced treadmill running (3 × 3 h with 30 min rest between the bouts) caused acute focal muscle damage in soleus muscles (see Irintchev & Wernig, 1987; Wernig *et al.*, 1990, 1991a). This damage



Figs 1 & 2. Double-stained cross-section from the endplate region of an intact soleus muscle labelled with rhodaminated α-bungarotoxin to visualize endplates (Fig. 1, rhodamine filter) and with J1/tn1 antibody against tenascin (Fig. 2, FITC filter). No tenascin immunoreactivity is detectable. Scale bar = 40 μm for Figs 1–4.

Figs 3 & 4. Double-stained cross-section from the endplate region of a soleus muscle seven days after sciatic nerve section. Tenascin immunoreactivity (Fig. 4) is associated with the endplates (Fig. 3). Additional spots probably located in degenerated nerve branches are visible. Arrows point to a muscle spindle in which a synaptic contact (Fig. 3) and tenascin (Fig. 4) are seen.

Figs 5 & 6. Double-stained longitudinal section from soleus muscle three days after denervation. A bundle of degenerated axons is tenascin positive (Fig. 6). Note that staining is confined to what appears as endoneurial tubes (one at long arrow), three of which reach endplates (Fig. 5, one at block arrow) where larger perisynaptic areas of tenascin immunoreactivity are present (Fig. 6, block arrow). Scale bar = 40 μm.

Table 1. Tenascin immunoreactivity (IR) and signs of muscle fibre damage in the endplate (middle) region and in the non-endplate (proximal) region of soleus at various times after denervation, paralysis with botulinum toxin and enforced running (3 × 3 h on one day)

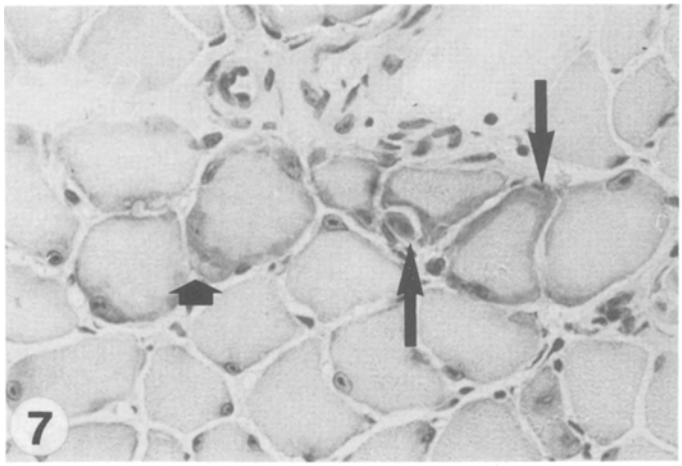
Days after treatment	*Number of endplates examined*	*Endplates with perisynaptic IR number (%)*	*Immunoreactivity in endplate region of soleus*	*proximal*	*Signs of muscle fibre damage*	
					acute	*chronic*
Denervation						
0.5	70	0 (0%)	–	–	–	–
1	80	20 (25%)	–	–	–	–
2	50	50 (100%)	–	–	–	–
3	73	73 (100%)	–	–	–	–
3	50	50 (100%)	–	–	–	–
7	75	75 (100%)	–	–	–	–
14	50	50 (100%)	–	/	–	–
21	50	50 (100%)	–	/	–	–
31	50	50 (100%)	–	/	–	–
Paralysis with botulinum toxin						
0.5	76	0 (0%)	–	–	–	–
1	28	0 (0%)	–	–	–	–
3	45	0 (0%)	–	–	–	–
3	50	0 (0%)	–	–	–	–
7	122	0 (0%)	–	–	–	–
7	86	0 (0%)	–	–	–	–
8	50	0 (0%)	+	–	+[1]	–
12	81	0 (0%)	–	–	–	–
Enforced running						
0	90	0 (0%)	+	–	+	–
0.5	113	7 (6%)	+	+	+	–
1	92	14 (15%)	+	+	+	–
2	132	21 (16%)	+	+	+	–
3	136	13 (10%)	+	+	+	–
6	131	4 (3%)	+	+	+	–
7	55	2 (4%)	+	+	+	–
14	103	0 (0%)	±	–	+	+
15	68	0 (0%)	–	–	–	+
21	72	0 (0%)	–	–	–	+

Data in each line are from a single muscle. Between 6 and 12 spaced serial cross sections through the endplate region were evaluated per muscle (typically 10 sections at 90 μm intervals). Sections were double-stained for ACh receptors (rhodaminated α-bungarotoxin) and tenascin/J1 (biotinylated second antibody and FITC-streptavidin). For definition of proximal region, acute and chronic signs of muscle damage see Materials and methods.
+ 'present'; – 'absent'; ± 'weak fluorescence around few split fibres' / 'no observations'; [1] signs of muscle fibre damage obviously inflicted by penetration with the needle during toxin injection were seen in this muscle.

is repaired within two weeks via proliferation of satellite cells and thereafter only chronic signs of previous injury (split fibres and centralized nuclei) occasionally are detectable (Table 1, see also Wernig *et al.*, 1991a).

Strikingly, tenascin immunoreactivity was already present in damaged muscles when first tested 10 h after the onset of exercise. Tenascin expression reached a peak at 2–3 days post-exercise and remained detectable for several days (Table 1, Fig. 15). This time course approximately corresponds to the occurrence of degeneration/necrosis of muscle fibres after damage (Table 1, Fig. 15, see Kuipers *et al.*, 1983; Zerba *et al.*, 1990; Wernig *et al.*, 1991a). In contrast to denervation, tenascin was not specifically associated with endplates but with sites of focal muscle fibre damage in both the endplate (Figs 7–10) and the non-endplate region of the muscle (Figs 11–14, Table 1), and was confined to the extracellular space.

Enhanced NCAM expression in the damaged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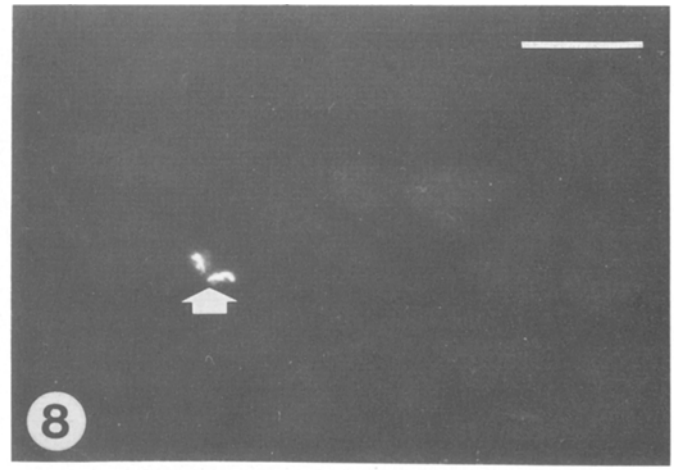
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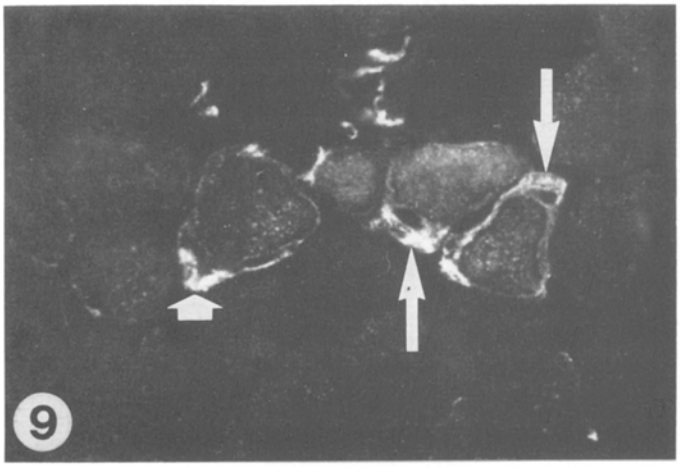
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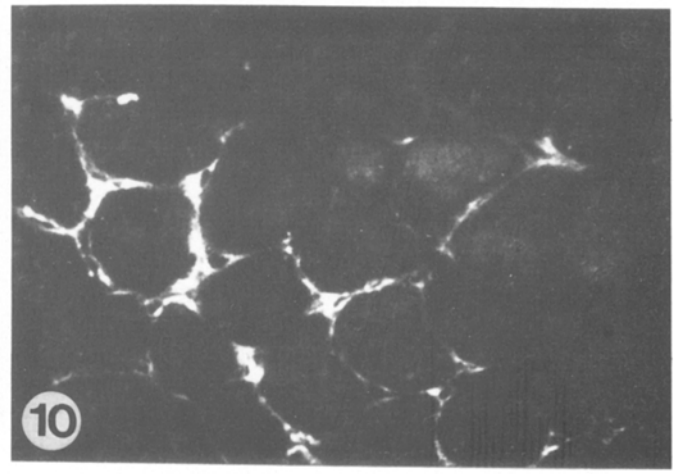
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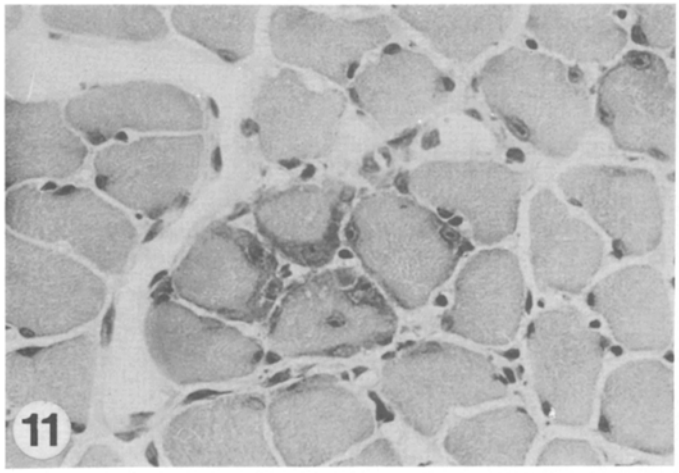
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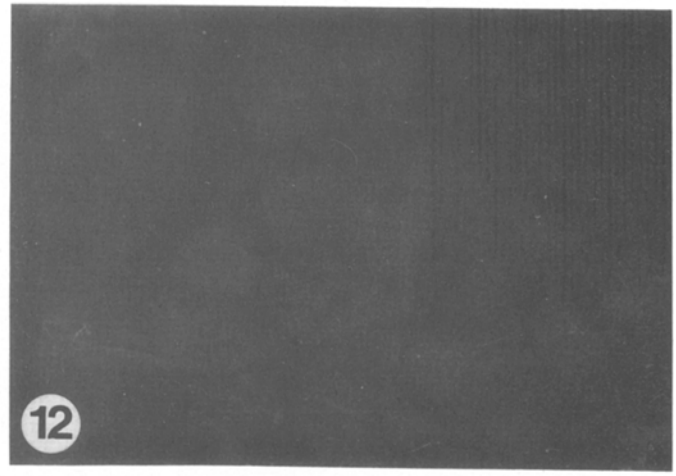
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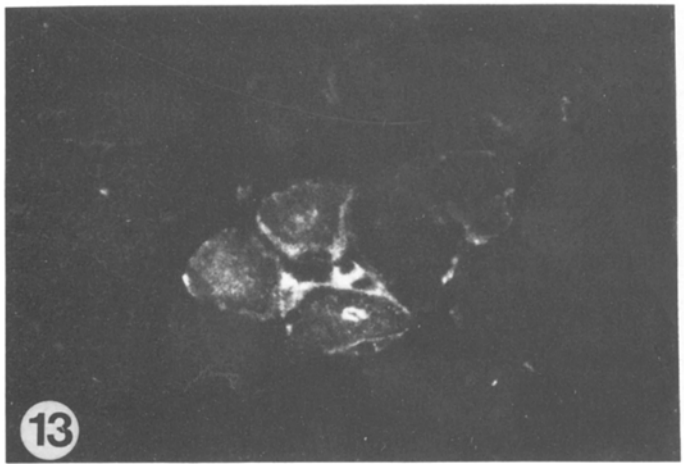
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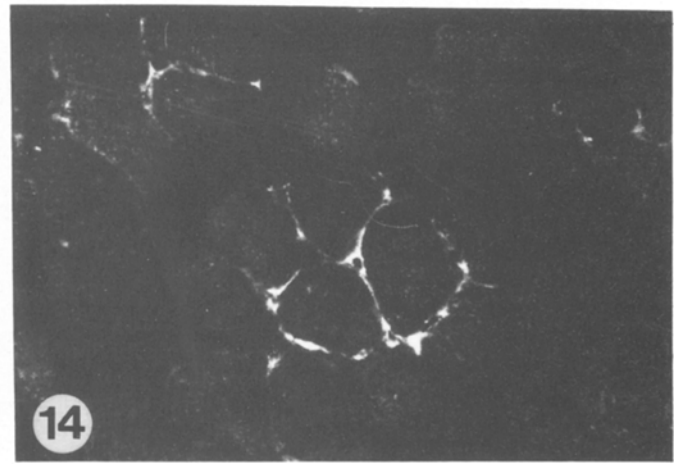
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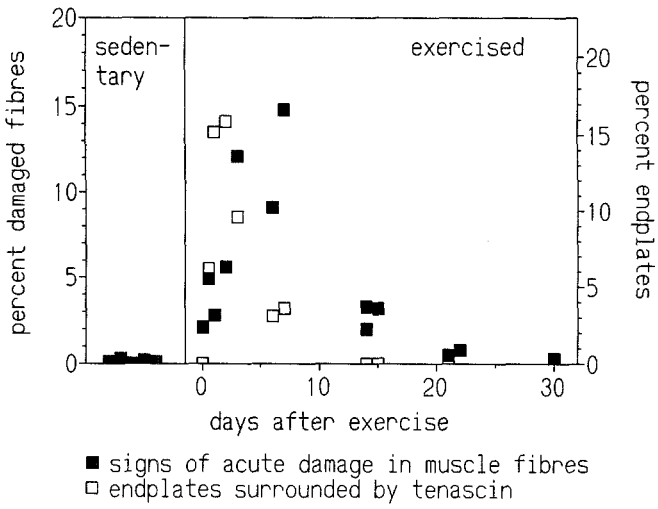

Fig. 15. Relative frequency of acute muscle fibre damage and endplates surrounded by tenascin immunoreactivity in individual soleus muscles after single exercise on a motor-driven treadmill. In muscles of intact sedentary mice (left panel) negligible amounts of damage and no tenascin are present.

muscles was obvious only at three days after the exercise and was associated with myogenic activity in the regeneration phase following muscle damage (Figs 9 & 13, see Irintchev & Wernig, 1987; Darr & Schultz, 1987; Cashman *et al.*, 1987). Colocalization of NCAM and tenascin in muscle cells or fibres was never observed (Figs 7–14). Both tenascin and NCAM were undetectable, except in association with structures that are normally positive (see *Normal muscles*), at the time when muscle repair was accomplished at about 14 days post-exercise.

Expression of tenascin and NCAM following exercise was also observed in the red portion of quadriceps muscle ($n = 3$), which is substantially damaged by exercise (see Vihko *et al.*, 1979). In contrast, extensor digitorum longus muscle, which does not suffer muscle damage (Irintchev & Wernig, 1987), did not show enhanced expression of tenascin or NCAM.

Muscle freezing

Expression of tenascin and NCAM were also found in soleus muscles 2–7 days after freezing ($n = 6$). Tenascin was present in areas of phagocytosis/regeneration as well as among preserved muscle fibre segments, and its expression obviously declined with time after cryodamage. NCAM was detectable in small cells, presumably myoblasts, two days after freezing and in larger profiles, myotubes and more mature muscle fibres, 3–7 days post-freezing. Numerous acid phosphatase positive phagocytes were present, as expected, in all cryodamaged muscles studied. To test whether this cell type, representing the vast majority of infiltrating cells in damaged muscles (see Discussion), expresses tenascin, we combined in the same sections acid phosphatase staining (with Naphthol AS-TR phosphate and para-rosanilin, red reaction product, see Wernig *et al.*, 1990) with anti-tenascin staining (with straptavidin-peroxidase and 4-chloro-1-naphthol, dark blue reaction product). Although some colocallization of the two reaction products was observed, most of the acid phosphatase positive cells were clearly negative for tenascin which strongly suggests, though does not completely exclude, that phagocytes do not contribute to the enhanced tenascin expression in damaged muscles.

Botulinum toxin paralysis

Muscle paralysis after local application of botulinum toxin did not cause detectable increase in tenascin expression (eight muscles studied 12 h–12 days after toxin injection, Table 1). In the same muscles NCAM increased and, as in denervated muscles, staining became detectable all along the length of the muscle fibres. NCAM immunoreactivity was first seen on the third day after botulinum toxin application. Four weeks after toxin injection ($n = 3$), when synaptic transmission is restored due to new synapse formation (Tonge, 1974; Wernig *et al.*, 1991b), extrasynaptic NCAM was no longer detectable in muscle fibres.

Figs 7–10. Near-serial cross sections from the endplate region of soleus muscle six days after a single running episode stained either with Toluidine Blue (Fig. 7), or anti-NCAM (Fig. 9), or double-stained with rhodaminated α-bungarotoxin (Fig. 8) and anti-tenascin-FITC (Fig. 10). Muscle fibres repairing from sublethal damage display intense peripheral basophilia in Toluidine Blue staining (fibres in the middle of Fig. 7) and in some of them moderate NCAM staining is seen at the fibre surface and in the sarcoplasm. Such fibres are often accompanied by basophilic satellite-like structures, at places clearly separated from the parent fibre (one at the middle arrow in Fig. 7), which show intense NCAM staining (arrows in Fig. 9) and also stain positive for other muscle markers (myofibrillar ATPase, glycogen phosphorylase, desmin, not shown). The satellite-like structures obviously arise from proliferation of muscle satellite cells; they have staining pattern similar to small regenerating fibres which could be observed either in clusters or singly (not shown). NCAM is also seen in an endplate (block arrow in Fig. 9) and several unmyelinated nerve fibres (upper third of Fig. 9). Tenascin is localized in the extracellular space surrounding damaged fibres and is not strictly confined to the endplate (Fig. 10). Scale bar = 40 μm for all figures in the plate.

Figs 11–14. Sections taken from the proximal part of the muscle shown in Figs 7–10. There are no endplates in this part of the muscle (Fig. 12) but muscle fibre damage (Fig. 11) as well as NCAM (Fig. 13) and tenascin (Fig. 14) are present.

Immunochemical analysis

In order to investigate the isoforms of tenascin expressed in muscle after the different experimental treatments, tissue samples of soleus muscle were analysed by SDS-PAGE and Western blot using polyclonal anti-tenascin antibodies. As observed by immunocytochemistry, enhanced expression of tenascin could be observed after muscle denervation, after enforced treadmill exercise or muscle cryodamage but not after muscle paralysis induced by local injection of botulinum toxin (Fig. 16). Tenascin expression upon enforced exercise appeared strongest after three days and decreased thereafter. Interestingly, the major isoforms of mouse tenascin were found re-expressed. Similar results were obtained from analysis of muscle tissue extracts and residual pellets, both of which contained about 50% of the detectable tenascin glycoproteins (Fig. 16 and results not shown).

Discussion

We show in this study, by both immunocytochemistry and Western blotting, that damage of axons or muscle fibres, but not muscle inactivity, induce tenascin re-expression in the adult neuromuscular system. Tenascin was expressed only in close vicinity of the damaged tissues indicating that the induction factor(s) are locally restricted. We could also demonstrate that irrespective of the type of tissue injury (nerve, muscle or both) the major isoforms of tenascin are re-expressed to an equal extent.

The finding of enhanced expression of tenascin at denervated endplates and in the denervated nerve confirm previous observations with polyclonal anti-J1, J1-200/220-specific monoclonal and anti-cytotactin antibodies (Sanes *et al.*, 1986; Daniloff *et al.*, 1989; Gatchalian *et al.*, 1989; Martini *et al.*, 1990). The distal to proximal course in tenascin re-expression coincided with the retrograde course of axon degeneration (Miledi & Slater, 1970; Manolov, 1974; Lunn *et al.*, 1990; present observations on acid phosphatase activity).

Focal muscle fibre damage can induce tenascin synthesis independent of nerve degeneration as seen in non-endplate regions of the muscle both after exercise and after muscle cryodamage. Together with the findings in denervated nerve, as well as observations of enhanced tenascin expression in skin wounds (Mackie *et al.*, 1988) and damaged monolayers of cultured fibroblasts (Gatchalian *et al.*, 1989), all results indicate that tenascin is induced in the course of cellular degeneration. This notion is further supported by the present finding that tenascin does not appear in botulinum paralysed muscles in which fibre atrophy but no degeneration/necrosis of muscle fibres or axons are observed (Duchen, 1970, 1971; present observations). Induction of enhanced tenascin synthesis as a result of tissue damage might be important for subsequent repair since tenascin appears to have mitogenic properties (End *et al.*, 1992).

The enhanced expression of NCAM along the entire length of denervated adult muscle fibres is well documented (Covault & Sanes, 1985; Daniloff *et al.*, 1986; Moore & Walsh, 1986). Similar upregulation is found after muscle paralysis with tetrodotoxin (Covault & Sanes, 1985) or botulinum toxin (present results) indicating that inactivity *per se* triggers NCAM expression. Inactivity produced by botulinum toxin did not, however, induce tenascin upregulation. In rat muscles paralysed with tetrodotoxin, Sanes and colleagues (1986) found J1-immunoreactivity with polyclonal antibodies which, however, could not dissociate by that time the tenascin-related form of J1 from other related molecules.

In damaged muscles, tenascin was confined to extracellular spaces and muscle cells obviously did not express this protein. Acid phosphatase-positive phagocytes (polymorphonuclear cells and monocytes/macrophages), which comprise the vast majority of cells infiltrating damaged muscles (Round *et al.*, 1987; Orimo *et al.*, 1991), appeared to be tenascin negative in double-stained sections. Most likely source of tenascin in the extracellular space of damaged muscles are the fibroblasts which have been shown to synthesize tenascin both *in vivo* and *in vitro* (Chiquet-Ehrismann *et al.*, 1986; Gatchalian *et al.*, 1989; Martini *et al.*, 1990). The short-term expression of tenascin in exercise-damaged muscles is, however, in marked contrast to the persistent synthesis by both fibroblasts and Schwann cells in denervated nerve stems (Sanes *et al.*, 1986; Gatchalian *et al.*, 1989; Martini *et al.*, 1990).

Immunochemical demonstration of tenascin glycoproteins in muscles subjected to different experimental treatments was possible by SDS-PAGE and Western blotting after extraction of the tissue with a buffer containing 2.0 M urea, similar to results obtained with the sciatic nerve (Martini *et al.*, 1990). Unlike with the sciatic nerve, however, only about 50% of tenascin glycoproteins were recovered in the muscle extract. This was not due to preferential solubility of individual tenascin isoforms because the extracts and the residual pellets contained tenascin glycoproteins with the same apparent M_r. Both after denervation and after enforced muscle training, enhanced expression of tenascin could be demonstrated. The known major isoforms of mouse tenascin, as judged by M_r comparison, were re-expressed, with no preponderance of individual M_r forms. In this respect, muscle tissue behaves like the sciatic nerve or the amphibian tail bud, where all known major isoforms could be detected in de- and regenerating tissue (Martini *et al.*, 1990; Arsanto *et al.*, 1990). On the other

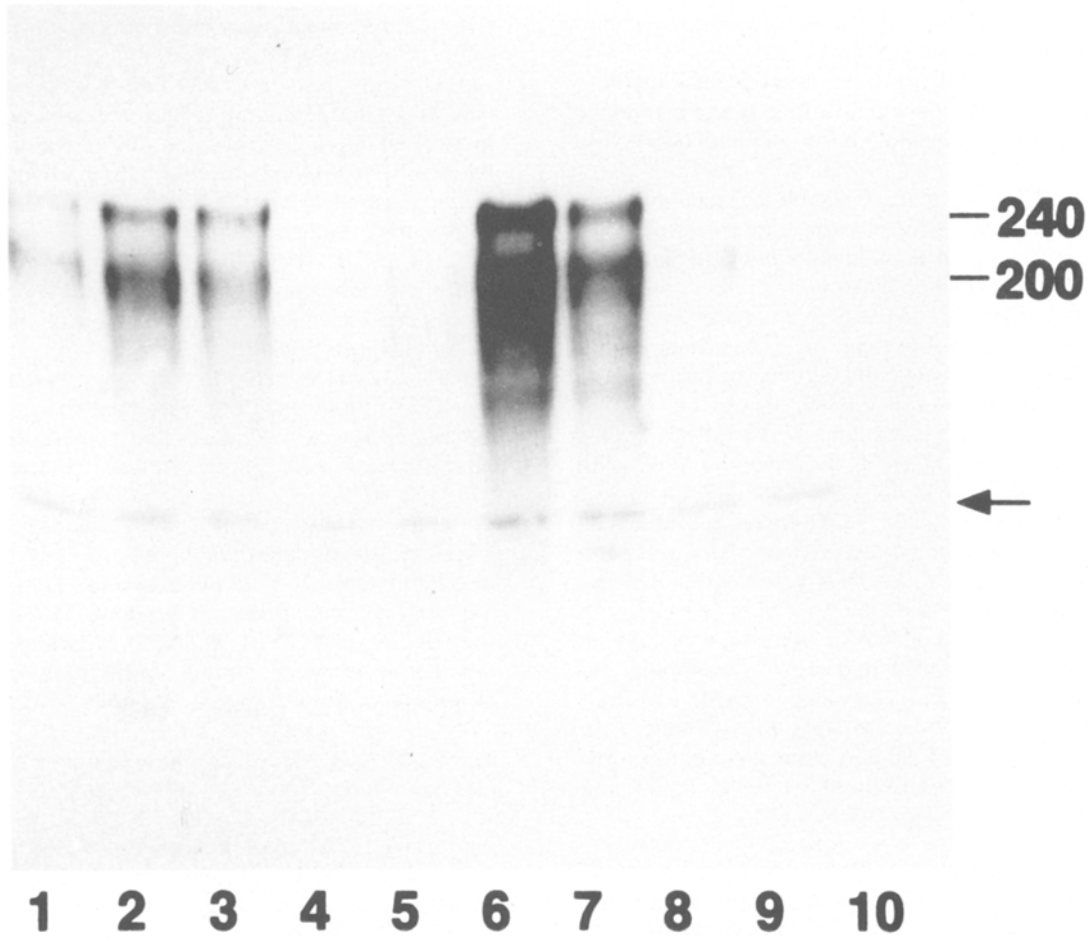

Fig. 16. Immunochemical analysis of tenascin glycoproteins in lesioned muscle tissues. Soleus muscles from the right (lanes 1–5) or from both the left and right limb (lanes 6–10) were processed for SDS-PAGE after diverse treatments as detailed below. Muscle pellets were resolved on 4–10% gradient gels, proteins were transferred to Immobilon membranes by Western blotting and filters were developed with pJ1/tn and ^{125}I-Protein A. An autoradiograph after 2 h of exposure is shown. The figure shows tenascin expression in soleus muscle five days after denervation (lane 1), three days after muscle freezing (lanes 2, 3), five days after paralysis with botulinum toxin (lanes 4, 5), three days (lanes 6, 7), seven days (lane 8) and 16 days (lane 9) after treadmill exercise, and without any treatment (control, lane 10). The arrow points to an unspecific band present at equal intensity in all samples and also detected by protein A alone (not shown). Molecular weights as indicated on the right were derived from co-migrating tenascin glycoproteins purified from postnatal mouse brain (not shown, Faissner & Kruse, 1990).

hand, it is known that tenascin isoforms of identical M_r could theoretically vary in the composition of alternatively spliced fibronectin Type III modules (Weller *et al.*, 1991; Siri *et al.*, 1991). For this reason, potential molecular variations might not be accessible to immunochemical analysis with polyclonal sera alone and require isoform specific probes which are not yet available for mouse tenascin.

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