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Differential expression of neural cell adhesion molecule (NCAM) after tenotomy in rabbit skeletal muscle

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Abstract

Tenotomy is a commonly encountered event in orthopaedic surgery. In 23 rabbit extensor digitorum longus (EDL) muscles, within 24 h after tenotomy, a marked drop in maximum force production occurred. This was not explainable based on architectural changes and histological examination using standard markers for muscle injury, i.e., haematoxylin and eosin morphology, developmental myosin heavy chain (MHC) immunolabeling, and quantitation of muscle fiber type percentage, area and distribution. The expression of neural cell adhesion molecule (NCAM), a glycoprotein expressed during muscle development was measured as a function of time in these muscles. NCAM expression was increased as early as one day after tenotomy with $2.2 \pm 1.2\%$ of the fibers showing positive expression. This expression level increased significantly to $15.4 \pm 15.2\%$ after 7 days and then subsided to $13.2 \pm 10.6\%$ 21 days after tenotomy. Two-way analysis of variance demonstrated a significant effect of time and a significant time x tenotomy method interaction. These results suggest that tenotomy leads to possible changes in muscle–nerve connections and/or excitation–contraction (EC) coupling. © 2002 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Tenotomy; Excitation–contraction coupling; Developmental myosin; Surgical tendon transfer; Muscle mechanics; Muscle injury

Introduction

Tendon laceration, surgical division, and rupture represent a spectrum of injury mechanisms that can be broadly characterized as tenotomy. While the surgical literature has necessarily focused on post-tenotomy tendon changes and methods available for tendon repair, concomitant muscle changes can also have dramatic functional effects. Tenotomy results in numerous muscular changes that have been reported both at the gross anatomical and molecular levels. Such changes include muscle fiber atrophy [4,10,16], increased perimysial and endomysial connective tissue [15], decreased serial sarcomere number [2,3], decreased force-generating capacity [5,10], increased contractile velocity [5], and altered expression of myosin heavy chain (MHC) [13] isoforms. We previously reported that a differential functional muscle response was observed when the tenotomy was imposed upon an activated muscle (''active tenotomy'', AT) compared to a passive muscle (''passive tenotomy'', PT) [1]. While a 50% decline in maximum tetanic tension (P_0) was observed one day after either AT or PT, muscles that were subjected to AT continued to weaken but ultimately mounted a more vigorous strengthening response compared to muscles subjected to PT (Fig. 1). Differences between group P_0 values were not explainable based upon quantitative analysis of muscle architecture, fiber size, or magnitude of regeneration as indicated by positive immunostaining with antibodies against developmental myosin [1]. Based upon our inability to explain contractile changes resulting from alterations in the contractile apparatus, we hypothesized that the force changes could result from disruption of the excitation–contraction (EC) coupling system within the muscle. The EC coupling system includes the electrical and subcellular events such as muscle fiber action potential conduction, transverse tubule conduction, ''activation'' of the dihydropyridine (DHP) receptors and calcium release in the vicinity of the myofilaments [9]. Precedent for selective disruption of EC coupling after muscle injury exists in the literature. Warren et al. [26] reported that eccentric exercise-induced injury could impair EC coupling in isolated mouse muscle and was further evidenced by the fact that

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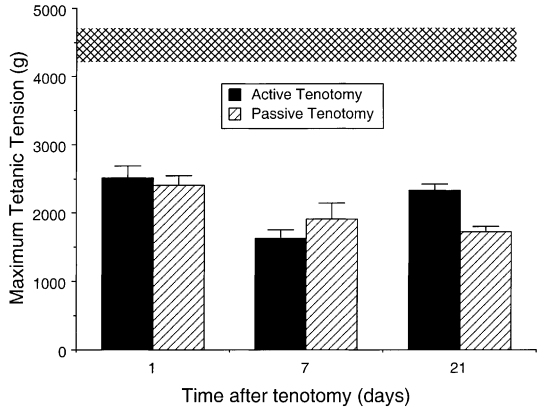

Fig. 1. Maximum isometric tension generated by EDL muscles after AT (filled bars) or PT (open bars). Two-way ANOVA revealed a differential effect on tenotomy based on the significant interaction term. Hatched bar represents maximum tetanic tension ± SEM of age-matched control EDL muscles (data from [1]).

torque production was uncoupled from the electromyographic signal [25]. We hypothesize that tenotomy leads to an analogous uncoupling of electrical impulses from mechanical force production in the early post-injury period. Further, should EC coupling be affected by tenotomy, upregulation of associated proteins could be observed such as acetylcholine receptors or neural cell adhesion molecule (NCAM). NCAM is upregulated after denervation [7] and regeneration [6] of adult skeletal muscle. In this report, we focus on NCAM, which is a cell-surface molecule that assists in establishing muscle–nerve and nerve–nerve connections during embryogenesis [12]. NCAM has been used to "quantify" muscle injury by determining the percentage of cells expressing the molecule during regeneration secondary to Bupivacaine injection [20]. Therefore, the presence of and time course of NCAM expression was studied in hopes of delineating molecular changes in skeletal muscle in the early post-tenotomy period. A brief version of this work has been presented [14].

Materials and methods

The rabbit extensor digitorum longus (EDL) was chosen for this tenotomy experiment due to its architectural similarity (multiple tendons from a common muscle belly, fiber length/muscle length ratio and pennation angle) to the human digital flexors and extensors which commonly experience tenotomy [17,18].

Experimental design

Muscles from a total of 23 male New Zealand white rabbits (mass 2.98 ± 0.27 kg) were studied, most of which were obtained from the previous study [1]. Negative controls experiments were performed on EDL muscles from untreated, size-matched animals on which no experimentation had been performed ($n = 2$). Positive controls, to test the specificity of the NCAM antibody were created by direct intramuscular Bupivacaine injection ($n = 2$) or denervation of the common peroneal nerve ($n = 2$). Seventeen rabbits were subjected to either AT ($n = 8$) or PT ($n = 9$) as described below. Animal care adhered to the NIH Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the San Diego V.A. Medical Center Animal Use Subcommittee. After terminal experiments, all animals were euthanized by injection of ~1 ml of concentrated (160 mg/ml) pentobarbital sodium via the marginal ear vein.

Active or passive tenotomy

Rabbits were anesthetized with 4% Aerrane® in an inhalation box and then maintained on 1–2% Aerrane® at 1–2 l/min via a face mask. Animals were positioned supine with the left foot secured to a foot plate attached to the arm of a torque motor (Cambridge Technology Model 6400, Cambridge, MA) to measure dorsiflexion torque during muscle activation. Subcutaneous electrodes were placed near the peroneal nerve at the knee. Maximal torque was recorded under computer control using single twitches at increasing voltage until a plateau was reached (typically 5 V). Contractile tests were performed at 2–3 times maximal voltage to ensure complete activation of the EDL muscle. This method has been implemented in this laboratory and we have seen that activation itself neither causes muscle deterioration nor, specifically, NCAM expression. A longitudinal midline incision was made between the proximal and distal anterior ankle retinacula and the four slips of the EDL tendon isolated in the jaws of an iris scissors. For the AT group, during activation and after maximal contractile force was reached, the four EDL tendons were briskly cut with the iris scissors resulting in rapid proximal muscle retraction and force loss (see [1, Fig. 1]) while for the PT group, tendons were cut without muscle activation and only modest retraction occurred. Wounds were closed with subcuticular 5-0 Dexon® (Davis & Geck, St. Louis, MO) and dressed with a Nexaband dressing. Animals were monitored until awake and returned to unrestricted cage activity. No animals showed any signs of distress after these procedures and weight gain paralleled that observed for nonoperated control animal subjects.

Control experiments

Under sterile conditions, with anesthesia as described above, serial bilateral 3 cm skin incisions were made on the lateral aspect of the lower leg. The fascia of the tibialis anterior (TA) or underlying EDL was incised carefully to avoid any damage to the underlying muscle. A 25 gauge needle and syringe were used to infuse a total volume of 5.0 cc of 0.25% Bupivacaine into the belly of the right TA. A minimal amount was noted to seep down the exterior aspect of the leg. Fasciotomy was imposed on the anterior compartment in order to avoid iatrogenic compartment syndrome. Animals were sacrificed 7 days after injection. Denervation controls were created by simply exposing the common peroneal nerve through a lateral incision on the leg beneath the biceps femoris and excising a 10 mm portion of the nerve. Denervated animals were euthanized after 48 h and the TA and EDL excised, flash-frozen in isopentane cooled by liquid nitrogen (−159°C) and stored at −80°C for subsequent histochemical processing.

Muscle isometric contractile testing

1, 7, or 21 days after tenotomy, animals underwent terminal muscle contractile testing. Anesthesia was induced and an anterior midline incision was made from mid-thigh to the ankle. The peroneal nerve was exposed and the leg was fixed to a rigid frame with 3.2 mm Steinman pins inserted through the distal femur and proximal tibia. The TA was cut distally and reflected. The proximal tendon stumps of the previously cut EDL were identified and secured to a servo-motor (Cambridge Technology Model 6400, Cambridge, MA). Muscle temperature was maintained at 37°C during testing with radiant heat, a mineral oil bath, and a servo-controller (Yellow Springs Instrument model 73A, Yellow Springs, OH). Isometric contractile properties were measured during twitch and tetanic contractions at stimulation frequencies from 5 to 200 Hz, of 600–800 ms duration, and a 90 s rest period interposed between successive tetani.

Stereological analysis

Serial transverse sections (6–8 μm) were cut at −20°C using a cryostat (Reichert–Junge, Model 9500) and mounted onto glass slides. Serial sections were stained with hematoxylin and eosin (H & E) for

observation of general muscle morphology and immunostained with antibodies against fast (MHC_f), slow (MHC_s) or developmental (MHC_d) MHC isoforms (Vector Laboratories, Burlingame, CA) and anti-NCAM (CD56) monoclonal antibody (Becton–Dickinson, San Jose, CA).

NCAM staining of denervated, Bupivacaine injected and tenotomized muscle revealed a continuum from light staining to very dark brown staining as indicated by the DAB chromagen. A single observer, blinded as to the identity of the tissue samples, analyzed the fibers under uniform conditions. Fibers were thus characterized as "negative", "positive", or "intermediate" based on staining intensity. No attempt was made to quantify optical density even though this would have been trivial since it is difficult to establish a linear relationship between immunostaining intensity and amount of protein present.

To obtain proper stereological sampling of fibers categorized canonically within the muscles, stereological equations were used to calculate the number of fields per section and the number of sections per muscle to be sampled [27]. Using the Bupivacaine-injected samples as an example for these calculations, we found that, sampling 10 fields per section and five sections per muscle, the percent positive staining with Bupivacaine injury was $15.0 \pm 1.9\%$ (mean $\pm$ S.D.). The standard deviation, σ, for this population of fields was 1.9, representing a coefficient of variation of 12.7%. Our experimental objective was to resolve a true difference between treatments of 20%, at a significance level, α, of 0.05 and with a statistical power, $(1 - \beta)$, of 0.8. Using standard equations [24] nine fields per section and three sections per muscle were calculated to be needed in order to achieve this level of confidence using this experimental design. This represented sampling of ~150 fibers per section and ~450 fibers per muscle.

Random coordinates were generated using a random number generator (StatView 5.0, Abacus Concepts, Berkeley, CA) and the fields corresponding to those coordinates acquired via on-line microscopy (Metamorph©, Universal Imaging Corporation, Westchester, PA). After acquisition, the number of positive- or intermediately stained fibers was counted within each section. To obtain proper stereological sampling (see above) of fibers within the muscles, stereological equations were used to calculate the number of fields per section and the number of sections per muscle to be sampled [27]. These data were then analyzed by two-way analysis of variance (ANOVA; StatView 5.0, Abacus Concepts, Berkeley, CA) using time (1, 7, or 21 days post-tenotomy) and tenotomy type ("active" or "passive") as independent variables. To determine whether NCAM expression and muscle force were related, the percentage of NCAM positive fibers was regressed on P_0 by simple linear regression.

Results

Bupivacaine was markedly toxic to muscle fibers and, after 7 days, led to disruption of muscle fibers and infiltration of leukocytes as well as a subsequent regenerative response characterized by formation of myotubes and immature myofibers observed on H & E staining (Fig. 2(A)). Bupivacaine-injected muscles demonstrated pronounced expression of MHC_d (developmental myosin) in the regions of maximum myofiber disruption (Fig. 2(B)). MHC_d was localized within these regions of newly formed myotubes and immature myofibers. Serial sections stained for NCAM revealed positive staining in the same anatomical location as that of MHC_d. Interestingly, NCAM was also expressed in a surrounding population of morphologically normal muscle fibers (Fig. 2(C)). MHC_d was not expressed to any degree by immunohistochemical testing in the tenotomy groups at 1, 7, and 21 days after tenotomy. (Sampling was performed on at least six fields per muscle.)

NCAM was expressed in tenotomized rabbit EDL muscle at levels significantly above controls within one day after both active and passive tenotomy (Fig. 3). NCAM was expressed in a very small number of control fibers,. $84 \pm 1.2\%$ (Fig. 3(A)). Qualitatively, NCAM levels increased from barely detectable levels 1 day after tenotomy (Fig. 3(B)) to relatively high levels 7 and 21 days after tenotomy (Figs. 3(C) and (D)). NCAM labeling increased to $2.20 \pm 1.22\%$ one day after tenotomy across experimental groups (AT and PT). There was a further significant increase to $15.4 \pm 15.2\%$ after 7 days and a slight decrease to $13.2 \pm 10.6\%$ 21 days after tenotomy. Two-way ANOVA revealed a significant effect

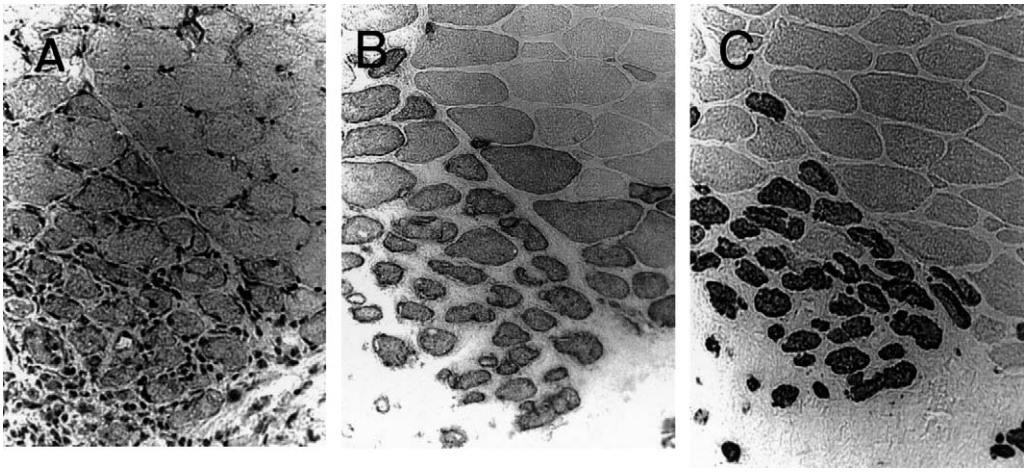

Fig. 2. Positive control serial light micrographs demonstrating reaction to Bupivacaine injection: (A) H & E stain demonstrates morphological signs of degeneration such as cellular infiltration and the presence of small, regenerating muscle fibers; (B) immunohistochemical stain for NCAM. Dark arrow points to regenerating fibers expressing NCAM while asterisk labels larger mature fibers, also expressing NCAM; (C) immunohistochemical stain for developmental myosin. White arrow points to regenerating fibers expressing developmental myosin that also express NCAM (Fig. 2(B)) while asterisk labels larger mature fibers, not expressing developmental myosin (i.e., not regenerating), but which did express NCAM (Fig. 2(B)).

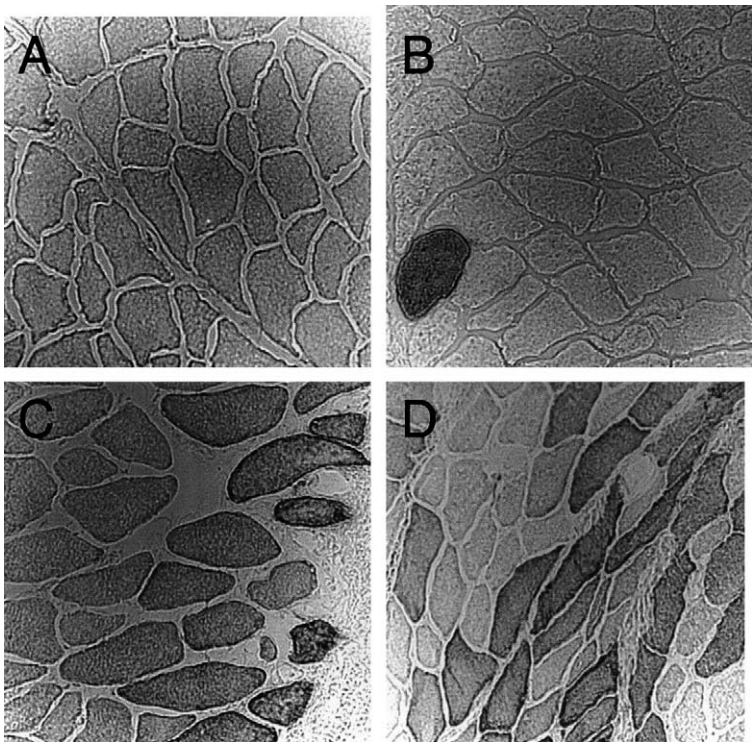

Fig. 3. Representative micrographs of rabbit EDL muscles immunostained for NCAM. Quantitative results from stereological analysis shown in Fig. 4: (A) control EDL muscle that received no treatment; (B) tenotomized muscle, one day after tenotomy; (C) tenotomized muscle, 7 days after tenotomy; (D) tenotomized muscle, 21 days after tenotomy. Darkly stained fibers are positive for the NCAM protein although the range in staining intensity varies among fibers. Arrows point to selected fibers expressing NCAM.

of time ($p < 0.01$), no significant effect of tenotomy type ($p > 0.3$) and significant tenotomy x time interaction ($p < 0.05$). These data demonstrate a time-dependent effect of tenotomy type, reminiscent of that observed for the functional data (Fig. 1). In spite of this significant interaction term, there was no significant relationship between percentage of NCAM positive fibers and force production as revealed by linear regression ($p > 0.6$). The absolute magnitudes of the effects (i.e., percentage change in force production and percentage of fibers expressing NCAM) were markedly different, clearly demonstrating a nonlinear relationship between the two phenomena.

NCAM staining was predominately localized to the peripheral borders of the sections examined in tenotomized muscles. The peripheral staining of fibers with NCAM was determined to be independent of an edge effect based on comparison of etched and non-etched serial sections of tenotomized muscle.

Discussion

Tenotomy is a common clinical entity that is relevant to multiple surgical subspecialties. Numerous studies have characterized structural and functional changes that occur secondary to tenotomy, but the precise mechanism by which these changes occur is not known. This is partly because functional studies have been performed independent of structural analysis.

A previous report on rabbit EDL and TA muscles demonstrated a functional difference between muscles that experienced active vs. passive tenotomy (Fig. 1). While it was not possible to account for such a change based upon measurement of the most likely structural parameters that could cause force differences [1], the current study reveals apparent interruption of normal muscle–nerve communication after tenotomy. This is indicated by the increased expression of NCAM, a molecule that is not normally expressed in adult muscle and which is upregulated after denervation and during myofiber regeneration.

Developmental and embryonic myosins are isoforms only expressed by developing but not adult muscle [8,11]. After various types of injury including contusion, eccentric contraction [19], and local anesthetic injury [20], there is also an increased expression of these isoforms. Immunohistochemical staining with a monoclonal antibody to human developmental myosin was used as a marker for regenerating fibers [8].

All tenotomized muscles in this study were analyzed using H & E and MHC_d staining, and revealed no morphologic changes that would suggest simple fiber atrophy, necrosis, inflammation or regeneration.

Architectural analysis revealed no difference in fiber area, fiber length (used to calculate cross-sectional area), or cross-sectional area between controls and tenotomized rabbits that could explain differences in force generating capacity [1].

To confirm the specificity of NCAM staining, we performed positive controls examining tissue after NCAM and MHC_d immunostaining and after H & E when muscles were either Bupivacaine injected or denervated (Fig. 2). Denervated muscle demonstrated no staining with MHC_d and diffuse staining with NCAM as expected and will not be discussed further. Bupivacaine injury led to an influx of inflammatory cells observable on H & E as well as visible destruction of myofibers and formation of a population of new myofibers. This area of the muscle corresponded on serial sections to the area of maximal expression of MHC_d where it specifically stained newly formed small myofibers in the midst of the inflammatory cells. The staining of this population of muscle fibers has been previously seen in the Bupivacaine damaged orbicularis oculi after one week [21]. Interestingly, NCAM stained this same population of newly formed small myofibers as well as a population of more peripheral normal-appearing large myofibers, not stained with MHC_d (Fig. 2). The population of normal-appearing fibers expressing NCAM may indicate that NCAM is a more sensitive marker for fiber injury compared to MHC_d, being expressed with a less significant injury to these myofibers.

There was also a qualitative difference between NCAM and MHC_d staining intensity after Bupivacaine-induced muscle injury. NCAM staining demonstrated a spectrum of intensity of staining while MHC_d was more definitive in intensity (Fig. 2). Whether this actually represents differences in the amount of each protein present is not addressed by this study due to the non-linear amplification of antibody staining that occurs with primary and secondary labeling of tissues.

NCAM staining in the Bupivacaine controls was located in the area of injection. In the tenotomized muscle, staining was maximal at the periphery of the muscle, although in some specimens it was uniform throughout the section. A common explanation for nonuniform distribution of staining across tissues is the "edge effect" – a manifestation of an apparent increase in staining intensity due to adhesion of staining liquids to tissue edge due to the cohesive forces of water [22]. To rule out this possibility, muscle cross-sections were etched along their length with a razor blade and then stained in the same fashion as the normal cross-sections. No increased staining was noted on the etched edges of the muscle while the outer surface of the muscle continued to stain in the same pattern for the NCAM.

NCAM expression after tenotomy is, to our knowledge, the first evidence of expression of a novel macromolecule in the tenotomy model (i.e., one expressed after tenotomy but not in normal adult skeletal muscle). The expression of NCAM within 24 h, too short a period for major structural reorganization or necrosis to occur, indicates a subtle physiologic response to tenotomy, possibly involving interaction between nerve and muscle. This effect appeared to be present both in active and passive tenotomy. In spite of the differential NCAM expression pattern, it was sufficiently different from the functional data (compare Figs. 1 and 4) such that no correlation existed between the type of tenotomy and the degree of NCAM expression ($p > 0.9$, $r^2 = 0.0026$). This lack of correlation probably indicates that NCAM expression is related to unloading phenomenon itself rather than the conditions of the tenotomy or the velocity of the shortening. The lack of quantitative agreement between the percentage of fibers expressing NCAM and the percentage change in maximum force indicates that there is not a simple one-to-one correspondence between fibers that express NCAM and force production. For example, even though only 2% of the fibers express NCAM one day after tenotomy, force decreased by 40%. We conjecture that these phenomena are associated with interruption in the EC coupling apparatus although not causally connected. Currently, it is impossible to detail the mechanistic basis for the relationship between NCAM expression and muscle force production but future experiments using measurement of single fiber mechanical properties may resolve this dilemma.

The 50% drop in tetanic tension after only 24 h with unchanged morphology and architecture argues strongly against major structural reorganizat ion causing functional changes. After a simulated tendon repair of the EDL to the extensor retinaculum at 7 days post-tenotomy, Abrams et al. [1] showed a rapid recovery of ten-

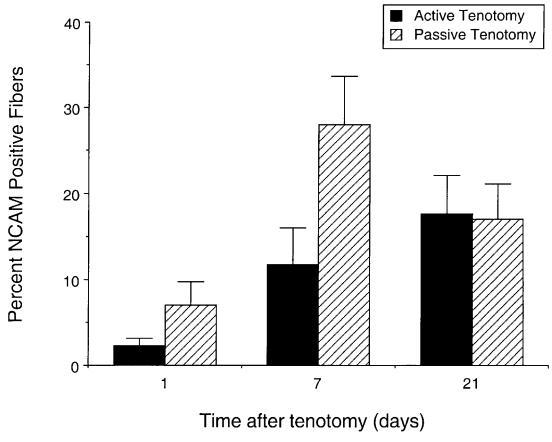

Fig. 4. Percentage of NCAM-positive cells in EDL muscles after AT (filled bars) or PT (open bars). Muscle correspond to those whose functional properties are shown in Fig. 1. Negative controls demonstrated $0.84 \pm 1.2\%$ positive fibers while positive controls (Bupivacaine injection) revealed $15.0 \pm 1.9\%$ positive fibers and is illustrated by the hatched bar.

sion after only 24 h of tendon reattachment. We hypothesize that the muscle itself sensed the chronic mechanical load change after tenotomy and theoretically uncoupled the EC coupling apparatus. This could have the effect of protecting the muscle from further damage which is known to occur when an unloaded muscle is permitted to actively contract [23]. This hypothesis could be tested by comparing the force generated after neural activation to that generated after direct activation of the myofibrillar apparatus using the skinned muscle fiber preparation in a manner similar to that recently reported after eccentric muscle injury [26].

A potential caveat in these results is that the skeletal immaturity of these rabbits may affect the muscle's response to tenotomy. While the long bone length is constant during epiphyseal closure, it is possible that the response of this young muscle does not represent the response of muscle in general. Further studies as a function of age are necessary to clarify this point.

Acknowledgements

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