Journal of
Clinical & Cellular Immunology

Renström et al., J Clin Cell Immunol 2013, 4:2
DOI: 10.4172/2155-9899.1000138

Research Article Open Access

TNF-alpha in an Overuse Muscle Model – Relationship to Muscle Fiber Necrosis/Regeneration, the NK-1 Receptor and an Occurrence of Bilateral Involvement

Lina Renström, Yafeng Song, Per S Stål and Sture Forsgren*

Department of Integrative Medical Biology, Anatomy Section, Umeå University, SE-901 87 Umeå, Sweden

Abstract

TNF-alpha is known to be involved in muscle damage and inflammation (myositis). The relationships between the TNF-alpha system and muscle fiber necrosis/regeneration and the tachykinin system in this situation are unclear. We have an experimental rabbit model related to unilateral muscle overuse which leads to marked muscle derangement and myositis bilaterally. Using this model, staining for TNF-alpha, in parallel with staining for the substance P-preferred receptor (NK-1R) and desmin were performed. Desmin staining was used as a reference concerning identification of degeneration/regeneration and the soleus muscle was the muscle examined. It was observed that the inflammatory cells, as well as blood vessel walls in the myositis areas, expressed TNF-alpha mRNA. Muscle fibers that were interpreted to represent necrotic fibers expressed TNF-alpha mRNA reactions and showed NK-1R immunoreactions, the reactions being confined to white blood cells that had infiltrated into the fibers. Muscle fibers that were interpreted to be in a regenerative state expressed patchy/widespread TNF-alpha mRNA and point like NK-1R immunoreactions. Abnormal muscle fibers thus showed TNF-alpha mRNA as well as NK-1R immunoreactions. Normal muscle fibers never showed these reactions. Occurrence of inflammatory cell and muscle fiber TNF-alpha mRNA reactions was equally marked in the myositis areas of the contralateral side as in these areas of the ipsilateral experimental side. The observations show that the TNF-alpha system is much involved in the processes that occur in the muscle derangement/myositis processes. The involvement relates to effects in processes of both regeneration and muscle fiber necrosis. It may be that substance P via activation through the NK-1R influences the TNF-alpha expression. The findings of TNF-alpha upregulation also for the contralateral side show that the TNF-alpha system is involved both ipsi and contralaterally during the development of myosits/muscle affection in response to unilateral overuse.

Keywords: Muscle; Inflammation; Myositis; TNF-alpha; Tachykinins; Degeneration; Regeneration

Introduction

TNF-alpha is a pro-inflammatory cytokine that drives the activation and recruitment of inflammatory cells, that maintains the inflammatory response and that is at the apex of the cascade leading to the production of various inflammatory mediators [1]. TNF-alpha is a therapeutic target for rheumatoid arthritis (RA) and several other autoimmune diseases [2].

One troublesome inflammatory condition is pronounced muscle inflammation (myositis). Via an established animal (rabbit) model for muscle overuse, we have recently observed that pronounced muscle derangement including a marked myositis develops in response to a combination of heavy exercise/electrical stimulation and injections of substances having pro-inflammatory effects [3,4]. The substances having these effects were delivered in the loose peritendinous tissue related to the tendon of the muscle in question (m. triceps surae). Previously, a marked myositis has been found to develop in muscle tissue for patients with inflammatory myopathies [5,6]. Experimentally, myositis is found to develop in response to crush-injury [7]. Damage of the muscle tissue can also occur in response to very marked overuse of skeletal muscle, i.e. in non-autoimmune conditions [8,9].

It has been previously suggested that an occurrence of a marked inflammatory response involving TNF-alpha can be of significance for the myositis processes that occur in the inflammatory myopathies [10,11]. It is also proposed that TNF-alpha can be directly responsible for damaging muscle fibres in these myopathies [12]. There is on the whole considerable evidence showing that TNF-alpha is produced in the skeletal muscle in inflammatory myopathies [6,12-16].

TNF-alpha is overexpressed in skeletal muscle in dystrophic mdx mice and blockade of TNF-alpha reduced the adverse responses to exercise-induced muscle damage seen in studies on these mice [17-20]. TNF-alpha is considered to be involved in the immune responses that occur after musculoskeletal trauma [21]. In our recent studies referred to above, TNF-alpha expression was detected at both protein and mRNA levels in inflammatory cells in the myositis process [4]. As seen in double-stainings, the TNF-alpha-expressing cells in myositis tissue were CD68 immunoreactive, i.e. corresponded to macrophages [4]. There were also TNF-alpha mRNA reactions in some of the muscle fibers of myositis areas. The importance of the TNF-alpha expression in this condition is, however, unclear. TNF-alpha is overall suggested to be involved not only in damaging events for skeletal muscle but also in the recovery of muscle function after traumatic muscle injury [22,23].

We have noted that marked overuse of the triceps surae muscle in rabbits not given injections of proinflammatory substances, also leads to affected muscles and myositis [24]. This fact was most obviously

***Corresponding author:** Sture Forsgren, Department of Integrative Medical Biology, Anatomy Section, Umeå University, SE-901 87 Umeå, Sweden, Tel: +46-90-7865147; Fax: +46-90-7866707; E-mail: sture.forsgren@anatomy.umu.se

Received January 16, 2013; **Accepted** March 18, 2013; **Published** March 25, 2013

Citation: Renström L, Song Y, Stål PS, Forsgren S (2013) TNF-alpha in an Overuse Muscle Model–Relationship to Muscle Fiber Necrosis/Regeneration, the NK-1 Receptor and an Occurrence of Bilateral Involvement. J Clin Cell Immunol 4: 138. doi:10.4172/2155-9899.1000138

seen when prolonging the experimental period (up to 6 weeks). This shows that myositis develops in our model without the requirement of pro-inflammatory injections. Interestingly, we noted that muscle derangement including myositis not only developed in the muscle on the experimental side but also in the muscle on the contralateral non-experimental side, suggesting effects via the nervous system [24]. We have also observed that there is a marked upregulation of the tachykinin system in this myositis process, including presence of the substance P (SP)-preferred receptor, the NK-1 receptor, both in degenerating and regenerating muscle fibers [25]. This can be of relevance when understanding the importance of the TNF-alpha system in this process. It is thus well-known that there are marked interrelationships between the TNF-alpha and tachykinin systems. For example, it has been shown that activation of mast cells and keratinocytes by SP leads to production of TNF-alpha [26], that tachykinins enhance the expression of TNF-alpha mRNA expression in monocytes isolated from RA patients [27], and that NK-1 receptor antagonists protect from TNF-alpha-mediated liver damage in mice [28]. It has also been shown that treatment with NK-1 receptor blocker substantially reduces the production of TNF-alpha into the temporomandibular joint cavity in experimentally induced inflammation for rats [29].

Based on what is described above it is evident that TNF-alpha is of importance in inflammatory myopathies and in the muscle for mdx mice. It is unclear what the effects of TNF-alpha are in non-autoimmune situations when there is marked muscle derangement and myositis, i.e. if TNF-alpha has effects during the processes of both muscle fiber necrosis and regeneration. Furthermore, there is no information as to whether there is a relationship between the TNF-alpha system and the tachykinin system in these situations. There is also no information on the possibility that TNF-alpha is involved in bilateral processes within muscle in response to overuse of muscle on only one side. In our previous study on soleus muscle using the overuse rabbit model (4), it was not studied as to whether there were correlations between the TNF-alpha expression pattern and features of degeneration/necrosis and regeneration and the tachykinin system and if there was a bilateral involvement of TNF-alpha in response to unilateral overuse.

In studies exploring the importance of TNF-alpha in the processes of degeneration/regeneration in muscle derangement and myositis we have here taken advantage of the fact that our overuse model leads to a marked muscle derangement including myositis and the fact that the tachykinin system is upregulated in this process. The overuse model for which no injection treatments were given was used. One muscle part of the triceps surae, the soleus muscle, was examined after 1,3 or 6 weeks of experiment (exercise/electrical stimulation) via the use of immunohistochemistry and in situ hybridization for the demonstration of TNF-alpha. In parallel, stainings for the SP-preferred receptor, the NK-1 receptor, were made. Furthermore, stainings for desmin, which can give information on degenerating/regenerating features, were applied. The soleus of both the experimental and contralateral sides was evaluated. The aim was to evaluate if the TNF-alpha system is involved bilaterally in the muscle derangement/myositis process, if there is a relationship between the TNF-alpha and tachykinin systems and to what extent TNF-alpha expression is related to muscle fiber necrosis and/or regeneration.

Materials and Methods

Animals

A total of 24 New Zeeland adult white female rabbits were used. The animals had an average weight of 4 kg and ranged in age from 6 to 9 months. They were divided into four groups consisting of six animals in each group. The animals of three of the groups were exposed to an experiment exercise procedure on their right leg, as described below. The animals of the fourth group served as controls and did not undergo any experiment at all (control animals).

Experimental procedures

The animals were exposed to an exercise procedure designed to lead to marked overuse of the triceps surae muscle and the associated tendon (the Achilles tendon). The procedure is performed according to previously described procedures [30], with some modifications [31]. All animals were anaesthetized during the exercise procedure, by means of an intramuscular injection of fentanyl-fluanisone (0.2-0.3 ml/kg) and diazepam (0.2 ml/kg; 5 mg/ml), followed by additional injections of fentanyl fluanison (0.1 ml/kg) every 30-45 min during the experimental procedure in order to maintain anaesthesia. For analgesia, buprenorphine (0.01-0.05 mg/kg) was given s.c. after each experiment session. The experiment lasted for 2 hours and was repeated every second day for 1, 3 or 6 weeks.

Design of the used apparatus

An apparatus ("kicking machine"), originally designed by Backman and collaborators for tendon studies [30] was used. This model has with some modifications been used for the induction of muscle affection including myositis [4,24,25] and for recent studies on the development of changes in the corresponding tendon, the Achilles tendon (tendinosis) [31]. The apparatus is constructed to generate passive flexion and extension of the ankle joint in one of the legs (the right leg). A pneumatic piston attached to the right foot produced the movements. During the plantar flexion, an active contraction was furthermore induced by electrical stimulation via surface electrodes (Pediatric electrode 40 426A, Hewlett Packard, Andover, MA, USA) placed 2 cm apart over the triceps surae muscle of the right leg. The optimal setting of movement has been found to be 9.5 cm, which gives a total range of motion of 55-65º for the ankle. A microswitch synchronized the stimulation unit (Disa stimulator Type 14E 10, Disa Elektronik A/S, Herlev, Denmark) with the plantar flexion movement of the piston. A single impulse with a duration of 0.2 ms was delivered 85 ms after the initiation of the plantarflexion at an amplitude of 35-50 V. The intensity was individually tested out to give powerful muscle contractions. The movement frequency was 150 movements per minute, i.e. 2.5 Hz. The left leg was not attached to the piston. The pelvis was strapped down and there were no ankle movements on the left side. In between the experiment periods, the rabbits were kept in ordinary cages, which allowed them freedom of movement. It was not obvious that they hereby showed amended movements or changed behaviours.

Sampling of specimens and sectioning

One day after the last exercise, the rabbits were sacrificed via an overdose of sodium pentobarbital and the entire triceps surae muscle with attached Achilles tendon was dissected out. Samples conforming to the soleus muscle part (5×8-10 mm) from both right and left sides were dissected out and fixed by immersion overnight at 4°C in an ice-cold solution of 4% formaldehyde in 0.1M phosphate buffer (pH 7.0). The samples were thereafter thoroughly washed in Tyrode´s solution containing 10% sucrose at 4°C overnight and mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, Ill.). The samples were then frozen in propane chilled with liquid nitrogen, and stored at -80°C. Series of 7-8 µm thick sections

were cut using a cryostat. The sections were mounted on slides precoated with chrome-alum gelatine and were then processed for immunohistochemistry. Other sections were processed for morphology or in situ hybridization.

Staining for demonstration of morphology (haematoxylin-eosin; H & E)

From all specimens, one section from the series of sections was stained in Harris Haematoxylin solution for 2 min. These sections were then rinsed in distilled water, dipped in 0.1% acetic acid for a few seconds, and then washed in running water. For counterstaining, immersion in eosin for 1 min was performed. The sections were finally dehydrated in ethanol and mounted in Permount.

Immunohistochemistry

TNF-alpha immunostaining: Sections of all specimens were processed for TNF-alpha immunohistochemistry. The sections were for this purpose initially pretreated with acid potassium for 2 min, a procedure used by the group to enhance specific immunofluorescence reactions [32]. Incubation for 20 min in a 1% solution of Triton X-100 (Kebo lab, Stockholm) in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, and three 5 min washes in PBS, then followed. Thereafter, the sections were incubated for 15 min in 5% normal donkey serum (code no: 017-000-121, Jackson ImmunoResearch Lab. Inc.) in PBS. Then, incubation with the primary TNF-alpha antibody, diluted in PBS (pH 7.4), was performed in a humid environment for 60 min at 37°C, after which washes in PBS 5 min×3 followed and another 15 min incubation in normal donkey serum. Next, the sections were incubated with either of these donkey antigoat IgGs for 30 min at 37°C: FITC-conjugated AffiniPure donkey antigoat IgG (705-095-147; Jackson ImmunoResearch Lab Inc, dilution 1:100) or Alexa FluorO 568 donkey anti-goat (Invitrogen, dilution 1:300). The sections were thereafter washed in PBS and then mounted in Vectashield Mounting Medium (H-1000) (Vector Laboratories, Burlingame, CA, USA). Examination was carried out in a Zeiss Axioscope 2 plus microscope equipped with epifluorescence optics and an Olympus DP70 digital camera.

Double-staining NK-1R/desmin: It was found to be of great interest to evaluate the reaction pattern of TNF-alpha mRNA in relation to that of NK-1 R and desmin immunostainings. Double-staining NK-1R/ desmin was therefore performed on parallel sections to those processed for TNF-alpha mRNA. Concerning staining for the NK-1R antibody, the procedures conformed to the procedures described above. That included the use of FITC-conjugated AffiniPure donkey anti-goat IgG (705-095-147) and rinsing in PBS 4×2.5 min, and as normal serum, 5% normal donkey serum in PBS was used. After the procedure for NK-1R immunolabelling was finished, the sections were rinsed in PBS 4×2.5 min, and incubated in 5% normal rabbit serum in PBS with BSA for 15 min. After that, the sections were incubated with the desmin antibody, diluted in PBS with BSA, in a humid environment. Incubation was performed for 60 min at 37°C. After incubation with this antiserum and 4x2.5 min washes in PBS, a new incubation in normal rabbit serum followed, after which the sections were incubated in rabbit anti-mouse immunolobulins/TRITC (R0276) (Dako, Denmark). The secondary antibody was used at a dilution of 1:40, and the incubation with this proceeded for 30 min at 37°C. The sections were thereafter washed in PBS for 4×2.5 min and were then mounted in Vectashield Mounting Medium (H-1000) or Mounting Medium with DAPI (H-1500) (Vector Laboratories, Burlingame, USA) in order to identify nuclei.

Antibodies and control stainings

An antibody against TNF-alpha produced in goats was used (AF-210-NA; R and D Systems) at a dilution of 1:50. It is by the suppliers described that this antibody is directed against *E. coli*-derived recombinant human TNF-alpha, the TNF-alpha specific IgG being purified by human TNF-alpha affinity chromatography. It is described to be specific via having the ability to neutralize the biological activity of recombinant human TNF-alpha. It is nevertheless shown that the TNF-alpha amino acid sequence homology between species is highly conserved and that TNF-alpha DNA sequence comparison shows an overall high sequence homology between various species (including rabbit) [33]. As control staining, preabsorption of the primary antibody with TNF-alpha antigen (T6674; Sigma; 20 µg/ml antiserum) was performed.

An antibody against NK-1R was furthermore used. It is produced in goats (sc-5220, Santa Cruz). It is an affinity purified polyclonal antibody raised against a peptide mapping within an internal region of NK-1R of human origin. It was regularly used at a dilution of 1:50-1:100 in 0.1% in PBS. Control stainings concerning this antibody included the use of NK-1R blocking substance (sc-5220P) (50 µg/ml antiserum).

An antibody against desmin was also used. It is a mouse monoclonal antibody (Ab D33, Dako, Denmark). It is by the supplier reported to be specific for desmin and to not show reactivity with other types of intermediate filaments. It was used at a dilution of 1:100 in PBS/BSA. This antibody has been evaluated in previous studies [24].

Control stainings regularly included stainings when the respective primary antibody was omitted.

In situ hybridization (ISH)

A digoxigenin (DIG)-hyperlabeled oligonucleotide probe (ss-DNA) for detection of TNF-alpha mRNA was used. The procedures were performed according an established protocol [34], using an alkaline phosphatase-labeled anti-DIG antibody (GeneDetect) for detection [4,35,36]. The anti-sense sequence of the probe used was CGGCGAAGCGGCTGACAGTGTGAGTGAGGAGCACGTAG-GAGCGGCAGC. The probe for TNF-alpha mRNA was used at 50 ng in 15 µl of hybridization solution.

The specimens used for ISH staining were from the 1week group (2 samples; one from exercised side, one from contralateral side), the 3 week group (2 samples; one from each side) and the 6 week group (2 samples; one from each side) and 3 samples from muscles of non-exercised animals. The samples were chosen in order to depict representative features for abnormal/normal muscle.

The tissue specimens were cut into 10 µm thick fresh cryosections using a cryostat (with a knife washed in 70% EtOH in DEPC-H2O) and mounted onto Super Frost Plus slides (nr.041300, Menzel Gläser, Braunschweig, Germany). The protocol that thereafter followed was that previously used in our laboratory for detection of mRNA for other substances [4,35-37].

An alkaline phosphatase (AP)-labelled anti-DIG antibody (Roche, Germany, 11 093 274 910) was used for detection. The sections were finally mounted in Pertex mounting medium. The corresponding sense DIG-hyperlabeled ssDNA probe was used as a negative control. As a positive control probe, a β-actin antisense probe (GD5000-OP) was used, comparisons being made with sense β-actin probe (GeneDetect, New Zealand).

Ethics

The study protocol was approved by the local ethical committee at Umeå University (A 34/07). The approval was obtained before the start of the study. A licensed breeder had bred all animals for the sole purpose of being used in animal experiments. All efforts were made to minimize animal suffering.

Results

Morphologic appearance

The structure of the muscle tissue was markedly changed in response to the experiment (Figure 1). That was especially the case in the 6w group, but was also to some extent seen in the 1 and 3w groups. The changes were seen for both the experimental and non-experimental (contralateral) sides and were noticeable for certain parts of the specimens ("myositis areas"). Other parts of the specimens showed in principle a normal morphology. The morphologic changes corresponded to an increase in loose connective tissue and muscle fiber alterations such as variations in fiber sizes and existence of frequent internal nuclei. There were also inflammatory infiltrates; white blood cells that were dispersed in the connective tissue areas. Marked muscle fiber alterations in the form of a massive infiltration of cells into the fibers were also seen (Figure 1c). The cells in these conformed to white blood cells, and have in a recent study been found to especially correspond to macrophages but also to some extent to eosinophils [24]. These fibers are further on referred to as necrotic muscle fibers. All these observations conform to observations made in parallel studies on soleus muscle specimens from the animals here analyzed [24].

TNF-alpha mRNA reaction in muscle of experimental animals

Via in situ hybridization, it was observed that the white blood cells of the inflammatory infiltrates frequently displayed TNF-alpha mRNA reactions. That was the case for the experimental (Figure 2a) as well as the contralateral (Figure 5) sides of the experimental animals. There were thus marked reactions for the cells after processing with antisense probe (Figure 2a) but not sense probe (Figure 2b).

There were also TNF-alpha mRNA reactions in blood vessel walls (Figures 3-5). That included the situation for the walls of veins (Figures 3 and 4) and small arterioles (Figure 5) and was noticeable for the experimental (Figures 3, 4a) as well as the contralateral (Figure 5) sides. The blood vessel wall reactions were restricted to blood vessels located in areas of the specimens showing morphologic affection, i.e. the myositis areas. Weak TNF-alpha mRNA reactions could be observed in fibroblasts (not illustrated).

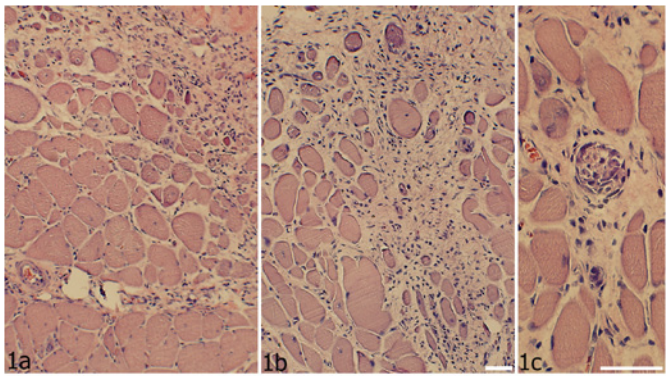

Figure 1: Sections of specimens stained with H&E. The specimens were from the experimental (a) and non-experimental (contralateral) (b,c) sides of the 6w group. It is shown that pronounced morphological changes had occurred. Marked presence of loose connective tissue and presence of inflammatory infiltrates (above, a, and above and to the right, b) and occurrence of a variability in muscle fiber sizes are thus seen. Presence of a morphologically abnormal muscle fiber (necrotic muscle fiber), being completely infiltrated by cells, is shown in (c) (middle part). Bars= 25 µm.

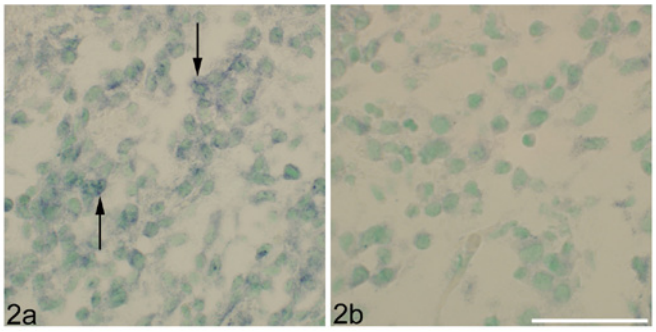

Figure 2: Sections of specimen processed for demonstration of TNF-alpha mRNA via in situ hybridization. Stainings with antisense (a) and sense (b) probes. The specimen was from the 6w group, experimental side. The regions shown in (a) and (b) are from corresponding areas. There are TNF-alpha mRNA reactions in white blood cells of an inflammatory infiltrate (arrows, a). There are no reactions in the sense control section (b). Bar=25 µm.

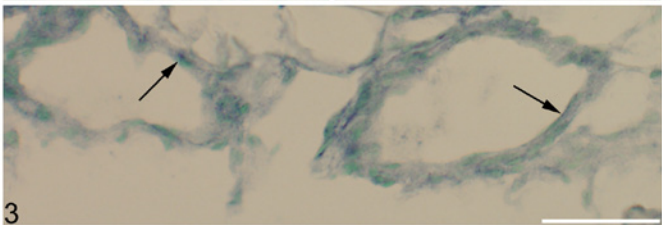

Figure 3: Section of specimen from the 6w group, experimental side, stained for demonstration of TNF-alpha mRNA; antisense probe. There are TNF-alpha mRNA reactions in walls of veins (arrows). Bar=25 µm.

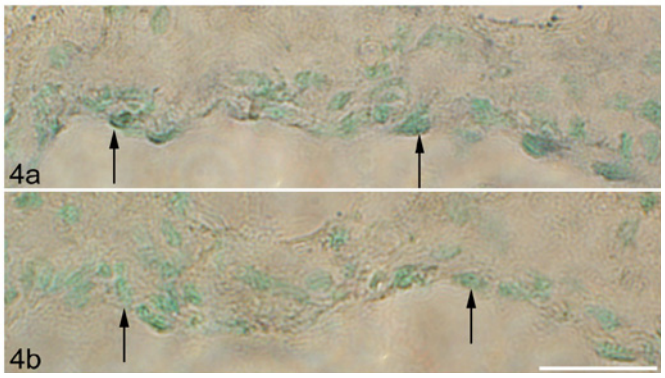

Figure 4: Sections of specimen stained for in situ hybridization (TNF-alpha mRNA); stainings with antisense (a) and sense (b) probes. The specimen was from the 3w group, experimental side. TNF-alpha mRNA reactions are observable in the endothelial part of a large vein. Arrows point at the occurrence of reactive (a) and non-reactive (b) endothelium of the large vein. Bar=25 µm.

Necrotic muscle fibers, i.e. fibers being completely infiltrated by white blood cells (c.f. above), exhibited TNF-alpha mRNA reactions: The reactions were seen for the infiltrated white blood cells (Figure 6). TNF-alpha mRNA reactions were also seen for certain other muscle

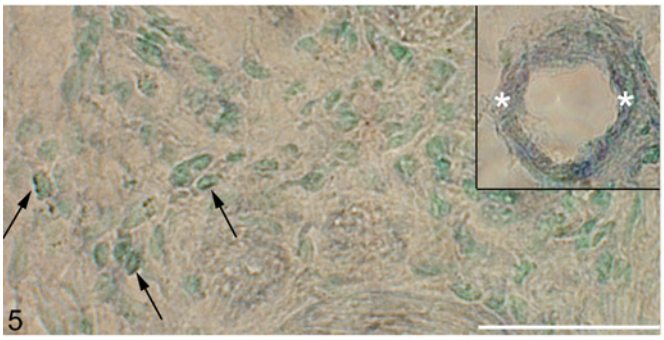

Figure 5: Section of specimen from the 6w group, contralateral side, stained for in situ hybridization (TNF-alpha mRNA); staining with antisense probe. TNF-alpha mRNA reactions are observable in cells of an inflammatory infiltrate (arrows), and in the wall of an arteriole (inset) (asterisks). Bar=25 µm.

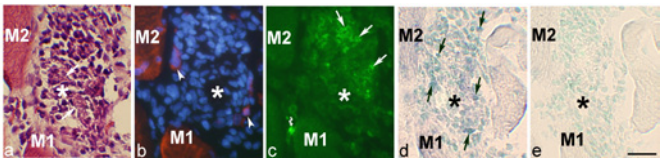

Figure 6: Sections from a series of sections of a specimen from the 6w group, experimental side. The sections were stained with H&E (a), for demonstration of desmin (including DAPI reaction) (b), NK-1R immunolabelling (c) and for demonstration of TNF-alpha mRNA (in situ hybridization) (antisense staining in d, sense staining in e). Asterisks mark a previously existing (necrotic) muscle fiber, in the area of which there is an accumulation of white blood cells [arrows in (a) point at debris material of the fiber]. There is no desmin immunoreaction within the fiber, whilst there are desmin reactions in the most peripheral cells arrowheads, which based on their strong desmin immunoreaction and occurrence of large nuclei are interpreted to be very small regenerating muscle fibers. There are cellular NK-1R immunoreactions in the necrotic muscle fiber (arrows). Artefact at curved arrow. Infiltrated cells do show TNF-alpha mRNA reactions (arrows in d). M1 and M2=corresponding muscle fibers. Bar=25 µm.

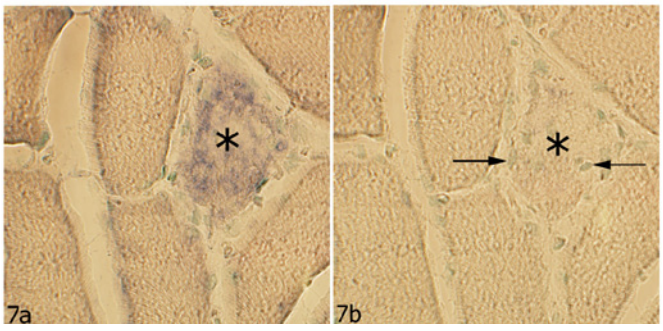

Figure 7: Sections processed for in situ hybridization. The specimen was from the 1w group, experimental side. The sections were processed with TNF-alpha antisense (a) and sense (b) probes. There are TNF-alpha mRNA reactions in muscle fibers (asterisks). The reactions occur in the form of patchy/widespread reactions (a). Arrows point at nuclei.

fibers. The reactions occurred in this case in the form of patchy and widespread reactions within the fibers (Figures 7 and 8). These muscle fibers exhibited frequently several internal nuclei (Figures 7-9). The fibers were frequently seen as grouped fiber profiles in the tissue (c.f. Figure 8) and are further described below. In the muscle fibers showing these patchy/widespread TNF-alpha mRNA reactions, cellular TNF-alpha reactions of the type seen in necrotic muscle fibers (c.f. Figure 6) were not detected.

The muscle fibers showing patchy or widespread TNF-alpha mRNA reactions as well as the necrotic muscle fibers were located to myositis areas of both sides of the experimental animals.

TNF-alpha mRNA reactions in normal muscle

Except for the occurrence of weak reactions for fibroblasts, TNF-alpha mRNA reactions were not noticed for any structure in the specimens of the non-experimental animals, i.e. the control animals. The situation was the same for normally appearing muscle tissue of the experimental animals. TNF-alpha mRNA reactions were thus never noticed for normal muscle fibers nor for blood vessel walls in normally-appearing muscle tissue. TNF-alpha mRNA reactions were not detected in nerve fascicles of control animals, nor in those of the experimental animals.

TNF-alpha in situ hybridization in relation to desmin and NK-1R immunohistochemistry

It was found relevant to further clarify the characteristics of the muscle fibers showing TNF-alpha mRNA reactions. The reaction patterns for muscle fibers processed for TNF-alpha mRNA using in situ hybridization were thus compared with immunoreactions for desmin. The use of sections processed for in situ hybridization and not for immunohistochemistry concerning TNF-alpha was related to the fact that specific TNF-alpha reactions were seen in the former sections but not in the latter (c.f. below). Desmin immunolabelling was performed as the pattern of desmin immunoreaction depicts features for degenerating/regenerating muscle fibers [38,39].

It was also considered to be of importance to clarify if there were relationships between the TNF-alpha mRNA and NK-1R immunoexpression patterns. It is thus known that SP regulates TNF-alpha transcription and release via activation of NK-1 receptors [26,40,41]. There are also other data which show that NK-1 receptors mediate the production of TNF-alpha [42].

It was found that the muscle fibers exhibiting patchy or widespread TNF-alpha mRNA reactions to a large extent exhibited a clearly stronger desmin immunoreaction than what was normally seen (Figures 9 and 10). That was the fact for entire muscle fibers or parts of muscle fibers. The characteristic striated desmin immunoreaction pattern normally seen for muscle fibers was not evident in the fibers/ fiber regions showing this strong desmin immunoreaction (Figures 9 and 10). Clumps of internal nuclei could be present in TNF-alpha mRNA expressing/strongly desmin immunoreactive muscle fibers (Figure 10a).

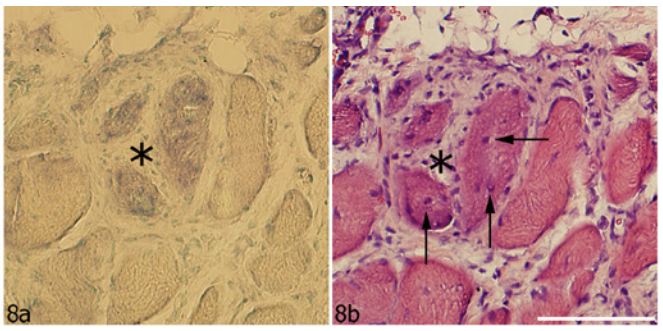

Figure 8: Parallel sections in a series of sections processed with TNF-alpha antisense probe (a) and stained with H&E (b). The specimen was from the 6w group, contralateral side. There are patchy/widespread TNF-alpha mRNA reactions in muscle fibers (asterisk). There is a presence of internal nuclei in these muscle fibers (arrows b). Bar=50 µm.

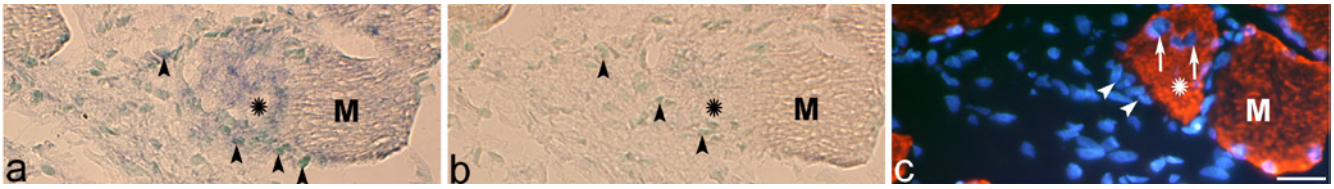

Figure 9: Parallel sections processed for TNF-alpha mRNA (antisense staining in a, sense staining in b) and desmin immunoreaction (c) (DAPI in mounting medium). The specimen was from the 6w group, experimental side. One of the muscle fibers (asterisk) shows a very strong non-striated desmin immunoreaction. There are intracellular nuclei (arrows, c) and patchy TNF-alpha mRNA reactions in this muscle fiber. There are also TNF-alpha mRNA reactions in cells located just outside this muscle fiber (arrowheads). There are no reactions in the sense control section (b). M=corresponding muscle fiber. Bar=25 µm.

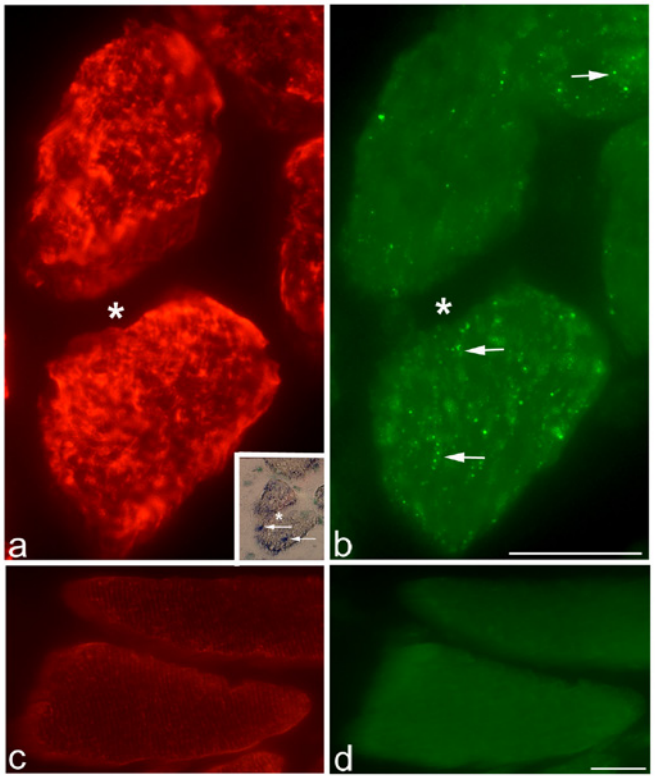

Figure 10: Specimen of soleus muscle, experimental side, 6w group. Parallel sections processed for desmin (a,c) and NK-1R (b,d) immunolabellings. Myositis area in (a,b) and normally appearing muscle area in (c,d). Muscle fibers in (a) show a strong and non-striated desmin immunoreaction pattern, whilst the muscle fibers in (c) show the characteristic desmin striated pattern. Point-like NK-1 R immunoreactions are detected in the fibers in (b) (arrows) but not in those in (d). In the inset in (a), the corresponding muscle fibers in a parallel section processed for TNF-alpha mRNA are shown in low magnification. There are patchy mRNA reactions (arrows). Asterisks at corresponding locations in (a-c). Bars=25 µm.

In previously existing muscle fibers (necrotic muscle fibers) and in which places there was an accumulation of white blood cells expressing TNF-alpha mRNA and only debris of muscle fiber material (c.f. above, Figures 6a and 6d) there was no desmin immunoreaction (Figure 6b). However, there was desmin immunoreaction in cellular structures in their peripheries and which we consider represent small regenerating muscle fibers (Figure 6b).

NK-1R immunoreactions were observed for both types of morphologically abnormal muscle fibers described above. NK-1R immunoreactions were thus detected within the necrotic muscle fibers, parts of the cells in these muscle fibers being NK-1R immunoreactive (Figure 6c). Furthermore, point like NK-1R immunoreactions were

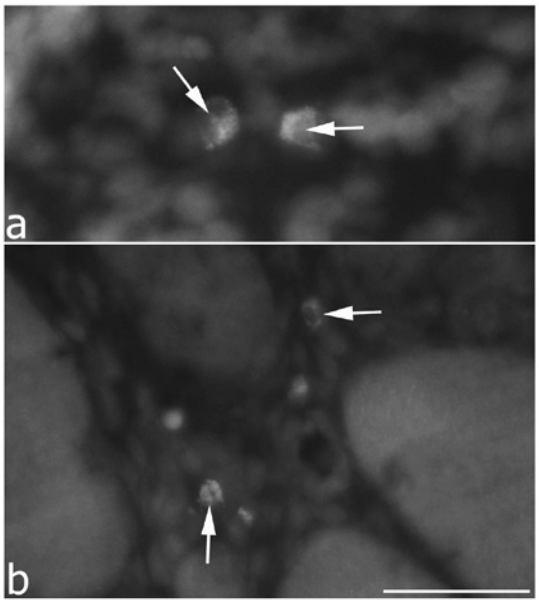

Figure 11: Sections of muscle specimens from the experimental (a) and contralateral (b) sides. The animals were from the 6w (a) and 3w (b) groups). There is TNF-alpha immunoreaction in cells of inflammatory infiltrates (arrows). Bar=25 µm.

detected in muscle fibers partly or entirely showing a strong desmin immunoreaction and patchy or widespread TNF-alpha mRNA reactions (Figure 10b). NK-1R immunoreactions were never detected in normally appearing muscle fibers (Figure 10d).

TNF-alpha immunohistochemistry

TNF-alpha immunoreaction was clearly detected in white blood cells that were dispersed in the tissue in myositis areas. That was the case for the experimental (Figure 11a) as well as the contralateral (Figure 11b) sides. There were very weak TNF-alpha immunoreactions in fibroblasts and a non-existence of TNF-alpha immunoreactions for the blood vessel walls and the nerve fascicles (not illustrated). There was also no clear TNF-alpha immunoreactions in the morphologically abnormal muscle fibers.

Discussion

Summary of the findings

The occurrence of marked muscle derangement including myositis after an experimental length of up to 6 weeks of overuse, the experiment being performed for 2h every second day, makes our model useful in clarifying the relationships between TNF-alpha and degeneration/regeneration and the tachykinin system. The pattern for the TNF-alpha

system contralaterally in response to unilateral overuse could also be followed.

In these studies we have shown that there indeed is an involvement of the TNF-alpha system for the muscular tissue in response to excessive exercise/electrical stimulation. The involvement of the TNF-alpha system was especially obvious at the 6 week stage, but TNF-alpha expressions could to a certain degree also be seen at the 1 and 3 week stages. The study much extends our previous observations for the rabbit model when injections having proinflammatory effects were given [4]. Completely new information was obtained via the parallel stainings for desmin and NK-1R and via examinations of both the experimental and non-experimental sides.

TNF-alpha mRNA expression was detected in various types of morphologically abnormal muscle fibers, but never in normally appearing muscle fibers. We also show that there is a relationship between the TNF-alpha and tachykinin systems concerning the affected muscle tissue. There was thus labelling for both TNF-alpha (at the mRNA level) and NK-1R (at the protein level) in morphologically abnormal muscle fibers, the significance of which will be discussed below. We have previously observed that there are NK-1R expressions also at the mRNA level for these morphologically abnormal muscle fibers [25]. Another noteworthy observation was the finding of a pronounced involvement of the TNF-alpha system for both sides. There is thus an involvement of this system both ipsi- and contralaterally during the development of the myositis/muscle affection.

As was observed previously [4], it is obvious that muscle fibers, blood vessel walls and to some extent fibroblasts clearly display TNF-alpha expression at the mRNA level but not clearly so at the protein level. This observation suggests that the production level of TNF-alpha for these structures is low, that our immunohistochemical method is not sensitive enough and/or that our in situ hybridization method detects small quantities of TNF-alpha mRNA. Nevertheless, the TNF-alpha mRNA reactions for abnormal muscle fibers and blood vessel walls of myositis areas were distinct and were in all cases verified via parallel staining with antisense and sense probes.

TNF-alpha in relation to muscle fiber necrosis/regeneration and NK-1R

A noteworthy finding was that TNF-alpha mRNA was detected in muscle fibers showing either a desmin loss or partially or entirely a strong desmin immunoreaction. The former fibers were completely filled with white blood cells and which we, based on the desmin immunoreaction pattern and the appearance after staining for htx-eosin, consider represent necrotic muscle fibers. It is thus known that desmin is undetectable or very much decreased in such muscle fibers [39]. The TNF-alpha mRNA reactions seen in this case were localized to the infiltrated white blood cells.

The TNF-alpha mRNA expressing muscle fibers which completely or to a large extent exhibited a strong desmin immunoreaction are likely to be in a regenerative stage, as it is well-known that there is an overexpression of desmin during muscle regeneration processes [38]. The TNF-alpha mRNA reactions in these fibers showed a patchy or widespread reaction pattern and not the cellular reaction pattern seen for the necrotic fibers (c.f. Figure 6d). It is thus obvious that there is an involvement of the TNF-alpha system both for the processes that occur in muscle fiber necrosis, TNF-alpha expression in this case being present for the infiltrating cells, and those of regeneration.

Interestingly, in muscle fibers in which TNF-alpha mRNA reactions were detected, NK-1R immunoreactions were also encountered. Thus, in fibers showing necrotic features, there were NK-1R immunoreactions in infiltrated white blood cells. In fibers which showed regenerative features, NK-1R immunoreactions occurred as point-like reactions. These findings show that the TNF-alpha and tachykinin systems are expressed in parallel in both degenerating/necrotic and regenerative muscle fibers.

It may be that NK-1R activation during the stages of degeneration/regeneration induces TNF-alpha expression. As described in the Introduction, there are thus reports suggesting that it is via activation of the NK-1R that SP can regulate TNF-alpha transcription. Treatment with NK-1R blocker has furthermore been found to substantially reduce the production of TNF-alpha into the temporomandibular joint cavity in experimentally induced inflammation for rats [29] and cigarette smoke amplifies the production of TNF-alpha from macrophages via SP binding to the NK-1R [42]. It has also been shown that stress induces SP in neurons and in local cells [43] and that SP here by increases the number of TNF-alpha-positive cells in airway inflammation, as seen in studies using a murine experimental model [44], and that an increase of SP in immune cells together with an increased SP in the innervation can activate the production of TNF-alpha in the generation of chronic gastritis [45]. Information from cell culturing studies have shown that SP can stimulate the production of TNF-alpha in inflammatory cells [46,47]. It was also shown that SP leads to TNF-alpha mRNA expressions for various types of human cells, e.g. keratinocytes [48], fibroblasts from hip periprosthetic membrane [49] and dental fibroblasts [50].

Functions of the TNF-alpha system for the myositis process–general aspects

There is well documented evidence that TNF-alpha is a central cytokine in the inflammation that occurs in conditions like RA [1]. Effects of TNF-alpha can be related to proinflammatory effects, including activation of macrophages and induction of apoptotic and necrotic cell death [51], as well as induction of nitric oxide synthase [52]. The results presented in this study suggest that tachykinins play a role in the establishment of TNF-alpha expression for muscle fibers. It may actually be that NK-1R activation via SP stimulation leads to TNF-alpha expression, consequently amplifying the inflammatory response. In accordance with this suggestion, it is emphasized that stimulation of SP via the NK-1R has an important influence on TNF-alpha mediated events in the mouse skin [53].

An involvement of the TNF-alpha system has been noted in other inflammatory situations affecting the locomotor system in addition to those related to myositis and arthritis. TNF-alpha mRNA is for example highly expressed in inflamed equine tendons [54] and in the subacromial bursa specimens in patients with subacromial bursitis [55]. TNF-alpha is also detected in human tendon tissue, implying that it can play a role in tendinopathy [55-57].

Apart from having pro-inflammatory, detrimental and apoptotic/necrotic modulating effects, TNF-alpha can also have protective, anti-apoptotic and regenerative effects [58,59]. Accordingly, it has been suggested that TNF-alpha plays a role in muscle regeneration [6,60]. Our findings relating TNF-alpha to regenerating muscle fibers suggest that this is the case in our experimental model. It may be that the type, severity and stage of the muscle injury determine the role TNF-alpha has [61]. It is apparent that there is a parallel occurrence of degenerative and regenerative processes at the stage of the muscle affection process that we have studied. TNF-alpha is apparently involved in both the detrimental and the regenerative events.

Bilaterality

TNF-alpha expression was obvious in the muscle affection process that occurs both ipsilaterally and contralaterally. We have recently noted that there is also a bilateral involvement of the SP-ergic system in our model [25]. There is thus a marked expression of NK-1R in the cells of the inflammatory infiltrates as well as in the blood vessel walls and the nerve fibers of the myositis areas bilaterally in the triceps surae muscle [25].

It may be that there are influences via the nervous system that explain these symmetric bilateral effects. One possibility is that there is an influence on the afferent innervation in the experimental side that secondarily leads to a contralateral effect. In accordance with such a suggestion it is considered that contralateral increases in sensory neural activity can be of importance for the symmetrical spread of the joint affections in RA [62].

It has been shown that bilateral symmetric changes concerning sensory neuropeptides for the joints can become established experimentally [63] and that a unilateral stimulus gives contralateral responses which are likely to be mediated via bilateral neural pathways [64]. Allodynia/hyperalgesia is reported to occur contralaterally in response to inflammatory stimuli [65] and unilateral electrical stimulation induces interstitial cell proliferation in both the stimulated and contralateral muscles [66]. Interestingly, paw inflammation in response to injection of complete Freund´s adjuvant leads to increased levels of TNF-alpha in the injected muscle as well as in the contralateral non-inflamed paw [67]. It has also been reported that there are increased levels of both TNF-alpha and SP in the hindpaws of rats treated with formalin injections in the tail [68]. The fact that we in preliminary studies have noted occurrence of TNF receptors in nerves in myositis specimens strengthens a suggestion that there actually can be an involvement of the TNF-alpha system in nerve affection processes in the present model. Further studies are to be undertaken in order to lead to a more precise understanding of the affected nerves and their relationship with the TNF-alpha system and the symmetric bilateral effects seen in our model.

Concluding remarks

It is known that cytokines such as TNF-alpha become involved in inflammation processes in extremeties [69]. There is also evidence which favours that there are regulatory interactions between muscle and the immune system during phases of muscle regeneration [70]. Also other factors than TNF-alpha are likely to be involved in the myositis process. In addition to what we have seen concerning the tachykinin system [25], we have also e.g. noted expression of glutamate transporter and glutamate receptor in white blood cells in the myositis areas (3). We have also observed expression of IL-3 in these cells in the myositis areas (unpublished observations).

Based on the findings in the current study, it appears as if TNF-alpha is of marked relevance in processes where there is a myositis/muscle affection in response to pronounced overuse (resulting from excessive exercise coupled with electrical stimulation). The results suggest that TNF-alpha is of importance for processes in both muscle fiber necrosis and regenerative events. Stimulation via SP on NK-1 receptors may possibly be responsible for the upreglation of TNF-alpha that we see for muscle fibers showing features of being in necrotic and regenerating phases. TNF-alpha may be interacting with several signal substances in these cases. One such may be insulin-like growth factor, which is reported to exhibit a cross-talk with TNF-alpha for diseased skeletal muscle [71]. There is a symmetric bilateral affection of the muscular tissue using the model and, as seen in the present study, a symmetric and bilateral involvement of the TNF-alpha system. The possible occurrence of detrimental and apoptotic/necrotic effects in relation to survival- and recovery-promoting and anti-apoptotic effects of TNF-alpha in myositis/muscle derangement should be further studied.

Acknowledgments

The authors are grateful to Ms. Ulla Hedlund for excellent technical assistance at the laboratory, to Mr. Adrian Lamouroux and Ms. Fellon Robson-Long for excellent technical service concerning the animal model, to Dr. Clas Backman and Professor Ronny Lorentzon for co-operation on the animal experiments and to PhD Paul Kingham for going through the manuscript from the English language point of view. The Faculty of Medicine, Umeå University, the Swedish National Centre for Research in Sports (CIF), and the J.C. Kempe and Seth M. Kempe Memorial Foundations supported the study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There is no actual or potential conflict of interest including financial, personal or other relationships with other people or organizations.

References

1. Feldmann M, Brennan FM, Maini RN (1996) Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 14: 397-440.
2. Feldmann M, Maini RN (2003) Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. Nat Med 9: 1245-1250.
3. Spang C, Scott A, Danielson P, Lorentzon R, Forsgren S (2012) VGluT2- and NMDAR1-expression in cells in the inflammatory infiltrates in experimentally induced myositis: Evidence of local glutamate signaling suggests autocrine/paracrine effects in an overuse injury model. Inflammation 35: 39-48.
4. Forsgren S, Renström L, Purdam C, Gaida JE (2012) TNF-Alpha in the Locomotor System beyond Joints: High Degree of Involvement in Myositis in a Rabbit Model. Int J Rheumatol doi: 10.1155/2012/637452.
5. Tews DS, Goebel HH (1996) Cytokine expression profile in idiopathic inflammatory myopathies. J Neuropathol Exp Neurol 55: 342-347.
6. De Bleecker JL, Meire VI, Declercq W, Van Aken EH (1999) Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies. Neuromuscul Disord 9: 239-246.
7. Peterson JM, Feeback KD, Baas JH, Pizza FX (2006) Tumor necrosis factor-alpha promotes the accumulation of neutrophils and macrophages in skeletal muscle. J Appl Physiol 101: 1394-1399.
8. Dennett X, Fry HJ (1988) Overuse syndrome: a muscle biopsy study. Lancet 1: 905-908.
9. Barbe MF, Barr AE (2006) Inflammation and the pathophysiology of work-related musculoskeletal disorders. Brain Behav Immun 20: 423-429.
10. Efthimiou P (2006) Tumor necrosis factor-alpha in inflammatory myopathies: pathophysiology and therapeutic implications. Semin Arthritis Rheum 36: 168-172.
11. Kondo M, Murakawa Y, Harashima N, Kobayashi S, Yamaguchi S, et al. (2009) Roles of proinflammatory cytokines and the Fas/Fas ligand interaction in the pathogenesis of inflammatory myopathies. Immunology 128: e589-e599.
12. Tews DS, Goebel HH (1998) Cell death and oxidative damage in inflammatory myopathies. Clin Immunol Immunopathol 87: 240-247.
13. Fedczyna TO, Lutz J, Pachman LM (2001) Expression of TNFalpha by muscle fibers in biopsies from children with untreated juvenile dermatomyositis: association with the TNFalpha-308A allele. Clin Immunol 100: 236-239.
14. Kuru S, Inukai A, Kato T, Liang Y, Kimura S, et al. (2003) Expression of tumor necrosis factor-alpha in regenerating muscle fibers in inflammatory and non-inflammatory myopathies. Acta Neuropathol 105: 217-224.
15. Salomonsson S, Lundberg IE (2006) Cytokines in idiopathic inflammatory myopathies. Autoimmunity 39: 177-190.
16. Tournadre A, Miossec P (2007) Cytokine response in inflammatory myopathies. Curr Rheumatol Rep 9: 286-290.
17. Radley HG, Davies MJ, Grounds MD (2008) Reduced muscle necrosis and

long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment. Neuromuscul Disord 18: 227-238.

18. Waters FJ, Shavlakadze T, McIldowie MJ, Piggott MJ, Grounds MD (2010) Use of pifithrin to inhibit p53-mediated signalling of TNF in dystrophic muscles of mdx mice. Mol Cell Biochem 337: 119-131.
19. Machado RV, Mauricio AF, Taniguti AP, Ferretti R, Neto HS, et al. (2011) Eicosapentaenoic acid decreases TNF-α and protects dystrophic muscles of mdx mice from degeneration. J Neuroimmunol 232: 145-150.
20. Piers AT, Lavin T, Radley-Crabb HG, Bakker AJ, Grounds MD, et al. (2011) Blockade of TNF in vivo using cV1q antibody reduces contractile dysfunction of skeletal muscle in response to eccentric exercise in dystrophic mdx and normal mice. Neuromuscul Disord 21: 132-141.
21. Reikerås O (2010) Immune depression in musculoskeletal trauma. Inflamm Res 59: 409-414.
22. Warren GL, Hulderman T, Jensen N, McKinstry M, Mishra M, et al. (2002) Physiological role of tumor necrosis factor alpha in traumatic muscle injury. FASEB J 16: 1630-1632.
23. Karalaki M, Fili S, Philippou A, Koutsilieris M (2009) Muscle regeneration: cellular and molecular events. In Vivo 23: 779-796.
24. Song Y, Forsgren S, Yu J, Lorentzon R, Stål PS (2012) Effects on contralateral muscles after unilateral electrical muscle stimulation and exercise. PLoS One 7(12): e52230. doi 10.1371/0052230.
25. Song Y, Stål PS, Yu J, Forsgren S (2013) Marked effects of tachykinin in myositis both in the experimental side and contralaterally: Studies on NK-1 receptor expressions in an animal model. ISRN Inflammation doi/10.1155/2013/907821.
26. Ansel JC, Brown JR, Payan DG, Brown MA (1993) Substance P selectively activates TNF-alpha gene expression in murine mast cells. J Immunol 150: 4478-4485.
27. Brunelleschi S, Bordin G, Colangelo D, Viano I (1998) Tachykinin receptors on human monocytes: their involvement in rheumatoid arthritis. Neuropeptides 32: 215-223.
28. Bang R, Biburger M, Neuhuber WL, Tiegs G (2004) Neurokinin-1 receptor antagonists protect mice from CD95- and tumor necrosis factor-alpha-mediated apoptotic liver damage. J Pharmacol Exp Ther 308: 1174-1180.
29. Denadai-Souza A, Camargo Lde L, Ribela MT, Keeble JE, Costa SK, et al. (2009) Participation of peripheral tachykinin NK1 receptors in the carrageenan-induced inflammation of the rat temporomandibular joint. Eur J Pain 13: 812-819.
30. Backman C, Boquist L, Fridén J, Lorentzon R, Toolanen G (1990) Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. J Orthop Res 8: 541-547.
31. Andersson G, Forsgren S, Scott A, Gaida JE, Stjernfeldt JE, et al. (2011) Tenocyte hypercellularity and vascular proliferation in a rabbit model of tendinopathy: contralateral effects suggest the involvement of central neuronal mechanisms. Br J Sports Med 45: 399-406.
32. Hansson M, Forsgren S (1995) Immunoreactive atrial and brain natriuretic peptides are co-localized in Purkinje fibres but not in the innervation of the bovine heart conduction system. Histochem J 27: 222-230.
33. Rees GS, Gee CK, Ward HL, Ball C, Tarrant GM, et al. (1999) Rat tumour necrosis factor-alpha: expression in recombinant Pichia pastoris, purification, characterization and development of a novel ELISA. Eur Cytokine Netw 10: 383-392.
34. Panoskaltsis-Mortari A, Bucy RP (1995) In situ hybridization with digoxigenin-labeled RNA probes: facts and artifacts. Biotechniques 18: 300-307.
35. Danielson P, Alfredson H, Forsgren S (2007) In situ hybridization studies confirming recent findings of the existence of a local nonneuronal catecholamine production in human patellar tendinosis. Microsc Res Tech 70: 908-911.
36. Jönsson M, Norrgård O, Forsgren S (2007) Presence of a marked nonneuronal cholinergic system in human colon: study of normal colon and colon in ulcerative colitis. Inflamm Bowel Dis 13: 1347-1356.
37. Bjur D, Danielson P, Alfredson H, Forsgren S (2008) Immunohistochemical and in situ hybridization observations favor a local catecholamine production in the human Achilles tendon. Histol Histopathol 23: 197-208.
38. Gallanti A, Prelle A, Moggio M, Ciscato P, Checcarelli N, et al. (1992) Desmin and vimentin as markers of regeneration in muscle diseases. Acta Neuropathol 85: 88-92.
39. Young C, Lin MY, Wang PJ, Shen YZ (1994) Immunocytochemical studies on desmin and vimentin in neuromuscular disorders. J Formos Med Assoc 93: 829-835.
40. Cocchiara R, Bongiovanni A, Albeggiani G, Azzolina A, Geraci D (1997) Substance P selectively activates TNF-alpha mRNA in rat uterine immune cells: a neuroimmune link. Neuroreport 8: 2961-2964.
41. Dickerson C, Undem B, Bullock B, Winchurch RA (1998) Neuropeptide regulation of proinflammatory cytokine responses. J Leukoc Biol 63: 602-605.
42. Xu J, Xu F, Lin Y (2011) Cigarette smoke synergizes lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α secretion from macrophages via substance P-mediated nuclear factor-κB activation. Am J Respir Cell Mol Biol 44: 302-308.
43. Joachim RA, Cifuentes LB, Sagach V, Quarcoo D, Hagen E, et al. (2006) Stress induces substance P in vagal sensory neurons innervating the mouse airways. Clin Exp Allergy 36: 1001-1010.
44. Joachim RA, Sagach V, Quarcoo D, Dinh T, Arck PC, et al. (2006) Upregulation of tumor necrosis factor-alpha by stress and substance p in a murine model of allergic airway inflammation. Neuroimmunomodulation 13: 43-50.
45. Sipos G, Sipos P, Altdorfer K, Pongor E, Fehér E (2008) Correlation and immunolocalization of substance P nerve fibers and activated immune cells in human chronic gastritis. Anat Rec (Hoboken) 291: 1140-1148.
46. Nair MP, Schwartz SA (1995) Substance P induces tumor necrosis factor in an ex vivo model system. Cell Immunol 166: 286-290.
47. Cuesta MC, Quintero L, Pons H, Suarez-Roca H (2002) Substance P and calcitonin gene-related peptide increase IL-1 beta, IL-6 and TNF alpha secretion from human peripheral blood mononuclear cells. Neurochem Int 40: 301-306.
48. Dallos A, Kiss M, Polyánka H, Dobozy A, Kemény L, et al. (2006) Effects of the neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide and galanin on the production of nerve growth factor and inflammatory cytokines in cultured human keratinocytes. Neuropeptides 40: 251-263.
49. Qian Y, Zeng BF, Zhang XL, Jiang Y (2008) Substance P stimulates production of interleukin 1beta and tumor necrosis factor alpha in fibroblasts from hip periprosthetic membrane. J Arthroplasty 23: 581-585.
50. Yamaguchi M, Ozawa Y, Mishima H, Aihara N, Kojima T, et al. (2008) Substance P increases production of proinflammatory cytokines and formation of osteoclasts in dental pulp fibroblasts in patients with severe orthodontic root resorption. Am J Orthod Dentofacial Orthop 133: 690-698.
51. Vanden Berghe T, Denecker G, Brouckaert G, Vadimovisch Krysko D, D'Herde K, et al. (2004) More than one way to die: methods to determine TNF-induced apoptosis and necrosis. Methods Mol Med 98: 101-126.
52. Akopov SE, Simonian NA, Grigorian GS (1996) Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. Stroke 27: 1739-1743.
53. Costa SK, Yshii LM, Poston RN, Muscará MN, Brain SD (2006) Pivotal role of endogenous tachykinins and the NK1 receptor in mediating leukocyte accumulation, in the absence of oedema formation, in response to TNFalpha in the cutaneous microvasculature. J Neuroimmunol 171: 99-109.
54. Hosaka Y, Kirisawa R, Ueda H, Yamaguchi M, Takehana K (2005) Differences in tumor necrosis factor (TNF)alpha and TNF receptor-1-mediated intracellular signaling factors in normal, inflamed and scar-formed horse tendons. J Vet Med Sci 67: 985-991.
55. Blaine TA, Kim YS, Voloshin I, Chen D, Murakami K, et al. (2005) The molecular pathophysiology of subacromial bursitis in rotator cuff disease. J Shoulder Elbow Surg 14: 84S-89S.
56. Millar NL, Wei AQ, Molloy TJ, Bonar F, Murrell GA (2009) Cytokines and apoptosis in supraspinatus tendinopathy. J Bone Joint Surg Br 91: 417-424.
57. Gaida JE, Bagge J, Purdam C, Cook J, Alfredson H, et al. (2012) Evidence of the TNF-α system in the human Achilles tendon: expression of TNF-α and

TNF receptor at both protein and mRNA levels in the tenocytes. Cells Tissues Organs 196: 339-352.

58. Echtenacher B, Männel DN, Hültner L (1996) Critical protective role of mast cells in a model of acute septic peritonitis. Nature 381: 75-77.

59. Inoue Ji, Ishida T, Tsukamoto N, Kobayashi N, Naito A, et al. (2000) Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp Cell Res 254: 14-24.

60. Chen SE, Jin B, Li YP (2007) TNF-alpha regulates myogenesis and muscle regeneration by activating p38 MAPK. Am J Physiol Cell Physiol 292: C1660-C1671.

61. Tidball JG (2005) Inflammatory processes in muscle injury and repair. Am J Physiol Regul Integr Comp Physiol 288: R345-R353.

62. Kelly S, Dunham JP, Donaldson LF (2007) Sensory nerves have altered function contralateral to a monoarthritis and may contribute to the symmetrical spread of inflammation. Eur J Neurosci 26: 935-942.

63. Bileviciute I, Stenfors C, Theodorsson E, Lundeberg T (1998) Unilateral injection of calcitonin gene-related peptide (CGRP) induces bilateral oedema formation and release of CGRP-like immunoreactivity in the rat hindpaw. Br J Pharmacol 125: 1304-1312.

64. Shenker NG, Haigh RC, Mapp PI, Harris N, Blake DR (2008) Contralateral hyperalgesia and allodynia following intradermal capsaicin injection in man. Rheumatology (Oxford) 47: 1417-1421.

65. Chang YW, Tan A, Saab C, Waxman S (2010) Unilateral focal burn injury is followed by long-lasting bilateral allodynia and neuronal hyperexcitability in spinal cord dorsal horn. J Pain 11: 119-130.

66. Hudlicka O, Graciotti L, Fulgenzi G, Brown MD, Egginton S, et al. (2003) The effect of chronic skeletal muscle stimulation on capillary growth in the rat: are sensory nerve fibres involved? J Physiol 546: 813-822.

67. Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S (1997) Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol 121: 417-424.

68. Bianchi M, Martucci C, Biella G, Ferrario P, Sacerdote P (2004) Increased substance P and tumor necrosis factor-alpha level in the paws following formalin injection in rat tail. Brain Res 1019: 255-258.

69. Barbe MF, Elliott MB, Abdelmagid SM, Amin M, Popoff SN, et al. (2008) Serum and tissue cytokines and chemokines increase with repetitive upper extremity tasks. J Orthop Res 26: 1320-1326.

70. Tidball JG, Villalta SA (2010) Regulatory interactions between muscle and the immune system during muscle regeneration. Am J Physiol Regul Integr Comp Physiol 298: R1173-R1187.

71. Grounds MD, Radley HG, Gebski BL, Bogoyevitch MA, Shavlakadze T (2008) Implications of cross-talk between tumour necrosis factor and insulin-like growth factor-1 signalling in skeletal muscle. Clin Exp Pharmacol Physiol 35: 846-851.