J. Anat. (2006) **208**, pp459–470

REVIEW

The role of neutrophils in injury and repair following muscle stretch

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Abstract

Stretch injury to the myotendinous junction is a common problem in competitive athletes and those involved in regular physical activity. The major risk factor for recurrent injury appears to be the primary injury itself. Physicians, physical therapists, athletic trainers and athletes alike continue to search for optimal treatment and prevention strategies. Acute inflammation is regarded as the body's generalized protective response to tissue injury. An especially important and unexplored aspect of inflammation following injury is the role of inflammatory cells in extending injury and possibly directing muscle repair. It has been suggested that the inflammatory reaction, although it typically represents a reaction to damage and necrosis, may even bring about some local damage of its own and therefore increase the possibility for scarring and fibrosis. Limiting certain aspects of inflammation may theoretically reduce muscle damage as well as signals for muscle scarring. Here we focus on the role of neutrophils in injury and repair of stretch-injured skeletal muscle. A minimally invasive model that generates a reproducible injury to rabbit skeletal muscle is presented. We present a plausible theory that neutrophil-derived oxidants resulting from the initial stretch injury are responsible for extending the damage. An anti-CD11b antibody that blocks the neutrophil's respiratory burst is employed to reduce myofibre damage. An intriguing area that is currently being explored in our laboratory and others is the potential role for neutrophils to contribute to muscle growth and repair. It may be possible that neutrophils facilitate muscle repair through removal of tissue debris as well as by activation of satellite cells. Recent and ongoing investigations point to interleukin-6 as a possible key cytokine in muscle inflammation and repair. Studies to elucidate a clearer understanding of this possibility will be reviewed.
Key words macrophages; muscle; neutrophils; stretch; tendon.

The clinical problem

Muscle strain injuries are common in sports that involve high-intensity sprinting efforts, including football and track and field sprinting and jumping (Orchard & Best, 2002; Orchard & Seward, 2002; Woods et al. 2004). Muscles that are most susceptible to injury are the two-joint or biarticular muscles such as the rectus femoris and biceps femoris that act to control and co-odinate joint motion (Orchard & Best, 2002; Woods et al. 2004). In addition, these 'high-risk' muscles are typically composed of a high percentage of Type-II muscle fibres, suggesting that the fibre metabolic profile is important in determining their functional characteristics and risk for injury. Of all the common sports injuries, muscle strains have one of the highest incidences of re-injury (Orchard & Best, 2002). The recurrence rate for hamstring strains (the most common muscle strain) is around 12% in professional soccer players (Woods et al. 2004) and upward of 30% (cumulative recurrence rate for the remainder of the season) in professional Australian footballers (Orchard & Best, 2002; Orchard & Seward, 2002). The burden therefore becomes high when one considers the primary incidence rate together with the high risk for recurrence.

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Accepted for publication *9 December 2005*

Physicians, physical therapists, athletic trainers and athletes alike continue to search for optimal treatment and prevention strategies. An area of controversy and ongoing study is the role of stretching in preventing and treating injury. A recent systematic review of the literature using the six available published studies concluded that stretching was not significantly associated with a reduction in musculoskeletal injuries (Thacker et al. 2004). This meta-analysis combined five studies on pre-exercise stretching with one article on regular stretching outside of exercise to obtain an overall summary that stretching does not prevent injury (odds ratio (OR) = 0.93; CI 0.78–1.11). As with stretching, little evidence exists that documents a correlation between increased flexibility and reduced incidence of injury. Further study is clearly needed to address the importance of stretching in injury prevention. It may well be that regular stretching and pre-exercise stretching should be considered as different interventions and strategies for reducing risk for injury.

The clinical presentation of muscle strains is highly variable and ranges from the simple 'muscle pull' to the career-ending complete muscle tear. A spectrum of injuries therefore exists; however, basic science and clinical studies have illustrated that muscle fibre tearing, haemorrhage and oedema typically characterize these injuries. Compared with other soft tissues, normal skeletal muscle has an intermediate to slightly long T1 relaxation time and short T2 relaxation time on magentic resonance imaging (MRI). Normal muscle appears relatively hypointense on both T1- and T2-weighted sequences. In general, all soft tissue injuries are best depicted on T2-weighted images, which optimize contrast between oedema and haemorrhage and the adjacent normal muscle (Fig. 1). Muscle strains should not be confused with delayed-onset muscle soreness (DOMS), where individuals experience diffuse muscle pain, swelling and stiffness following an unaccustomed bout of eccentric exercise. In the case of DOMS, the recovery interval is often short and subsequent similar bouts of exercise typically produce fewer symptoms and clinical findings.

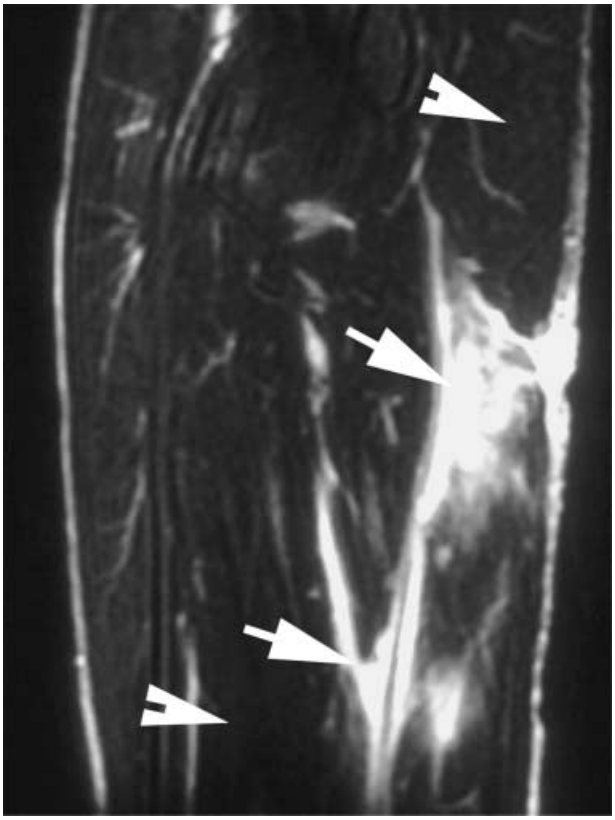

Fig. 1 Coronal T2 MRI image illustrating contrast between oedema (arrows) and adjacent normal muscle (arrowheads) in the stretch-injured biceps femoris muscle. These images were acquired 3 days following the injury.

Clinical studies of muscle strains

Not only can the initial injury be a source of time lost from sport, recreation and occupation, but their tendency to recur with chonic pain and disability is not infrequent. In fact, the major risk factor for recurrent injury appears to be the primary injury itself (Orchard & Best, 2002). In recent years, use of MRI for assessing severity of muscle strains and time for return to play has become more common in clinical practice. In general, the size of a hamstring muscle strain (defined by the cross-sectional area of involvement or longitudinal size on a T2-weighted image) correlates with convalescence time and provides some guidance for safe return to performance (Cross et al. 2004). A key component to complete recovery following muscle strains may be the control of muscle scarring and fibrosis (Hurme et al. 1991; Orchard & Best, 2002). Experiments using a muscle laceration model have shown improved recovery of contractile function when scarring is decreased though inhibiting TGF-β_1 formation (Kosonen et al. 2000; Sato et al. 2003). TGF-β_1 has been implicated in the pathogenesis of scarring and fibrosis of many tissues and similarly appears to play an important role in the muscle necrosis and fibrosis associated with Duchenne's muscular dystrophy (Rando, 2001). Although the exact mechanism for TGF-β_1-induced

fibrosis is not known, its presence leads to the differentiation of myoblasts and muscle-derived stem cells into a myofibroblastic lineage (Li & Huard, 2002). It is therefore conceivable that TGF-β_1 plays an important role in the limitation of muscle scarring although its utility in humans awaits further investigations.

An especially important and unexplored aspect of inflammation following injury is the role of inflammatory cells in extending injury and possibly directing muscle repair (Tidball, 1995). It has been suggested that the inflammatory reaction, although it typically represents a reaction to damage and necrosis, may even bring about some local damage of its own and therefore increase the possibility for muscle fibrosis and scarring (Tidball, 1995). Limiting certain aspects of inflammation may theoretically reduce muscle degeneration as well as signalling mechanisms for muscle scarring. By contrast, this approach may potentially reduce the availability of growth factors such as IGF-1 and cytokines that promote muscle regeneration.

The remainder of this review will focus on the role of neutrophils in injury and repair of skeletal muscle. A non-invasive model that generates a measurable and reproducible stretch injury to rabbit skeletal muscle is presented. Studies exploring the role of neutrophils in stretch injury to skeletal muscle will be reviewed. Data suggesting a possible role for neutrophils in muscle repair will be presented. Recent and ongoing investigations point to interleukin-6 (IL-6) as a possible key cytokine in muscle inflammation and repair. Studies to elucidate a clearer understanding of this possibility are reviewed.

Injury model

The majority of animal studies of stretch injury to skeletal muscle have employed rather invasive and non-physiological methods to create injury (Almekinders & Gilbert, 1986; Nikolaou et al. 1987). In particular, these models have not clearly demonstrated that a reproducible and quantifiable injury can be produced (Almekinders & Gilbert, 1986; Nikolaou et al. 1987). These limitations make it difficult to study and compare treatment strategies and perhaps necessitate unnecessarily large numbers of animals to detect quantifiable differences.

To study muscle stretch injury and repair in a controlled and reproducible way, it was necessary to develop a new animal model. We have modified an existing approach to generate an injury consistent with a typical muscle tear in humans where the greatest damage (fibre tearing, haemorrhage, oedema) occurs at or near the myotendinous junction (Best et al. 1998, 1999). In contrast to previous models of stretch injury to skeletal muscle (Almekinders & Gilbert, 1986; Nikolaou et al. 1987), our injury is produced using physiological joint and muscle motions. A custom-designed rotational potentiometer is coupled to a tendon-shortening device that accurately controls tendon shortening and resultant muscle damage (Fig. 2). This system results in a reproducible size and location of muscle injury that more accurately permits comparisons between groups and treatments using a minimum number of animals and specimens (Fig. 3). We have examined minor stretch injury using scanning electron microscopy (SEM) and noted fibre tearing in the distal muscle belly (Fig. 4a) and gap formations at the myotendinous junction (Fig. 4b). Interestingly, these observations were made in the face of 'normal' light microscopy, suggesting that SEM may be a more sensitive way of detecting smaller injuries, although the exact clinical relevance of these findings is still in question. In particular, there are no studies that compare the magnitude of injury (amount of damage) that has been produced in animal models with the extent of injury seen in humans. Moreover, despite our abilities to confirm an injury that is similar from animal to animal (Best et al. 1998), some caution should be

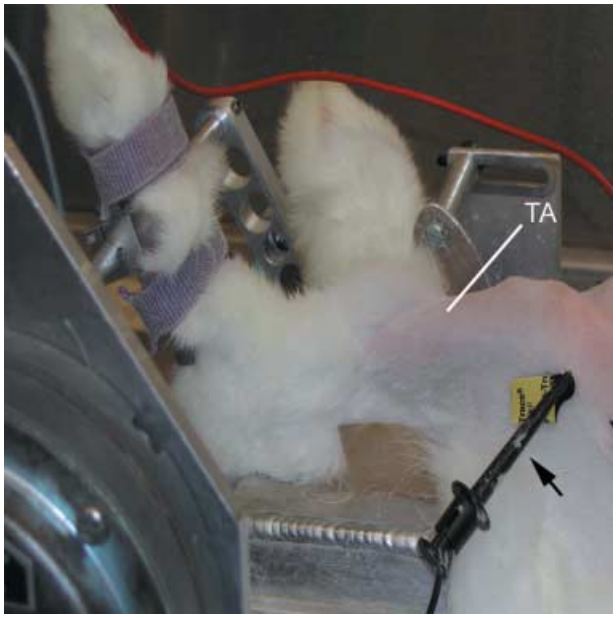

Fig. 2 Experimental set-up to produce stretch injury to the rabbit tibialis anterior (TA). Muscle stimulation is achieved by surface stimulation (arrowhead) while the foot is rotated into plantarflexion.

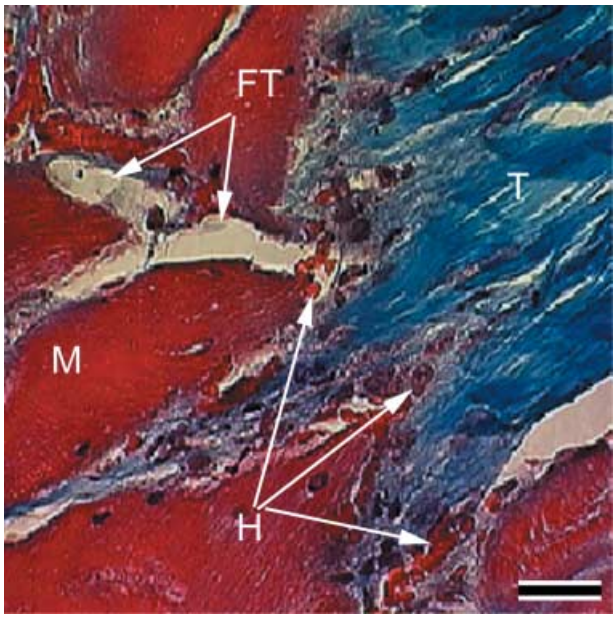

Fig. 3 Masson's stain depicting fibre damage and cellular infiltration at 24 h following injury to the rabbit tibialis anterior (TA). FT, fibre tearing; H, haemorrhage; T, tendon; M, muscle. Scale bar = 200 μm.

exercised given that our input parameters to create injury (e.g. muscle is tetanized and the ankle is forced into plantarflexion) are probably not occurring during such motions as sprinting or jumping when humans most often tear their hamstrings or quadriceps.

Inflammatory cell response to muscle stretch injury

Cellular infiltration of injured skeletal muscle progresses though several stages, generally characterized by early neutrophil invasion, followed by sequential increases in ED1$^+$ and ED2$^+$ macrophages (Tidball, 1995). The time course varies with species and model but in general follows a sequential invasion of the damaged tissue by these cell types that can persist for days to weeks. Previous studies of stretch injury have typically used general histological staining (e.g. haematoxylin–eosin) and muscle myeloperoxidase (MPO) activity (Best et al. 1998; Brickson et al. 2001) to identify leukocyte subtypes. These studies suggest that peak neutrophil infiltration of the damaged muscle occurs within 24 h post-injury and is associated with both maximum fibre tearing and

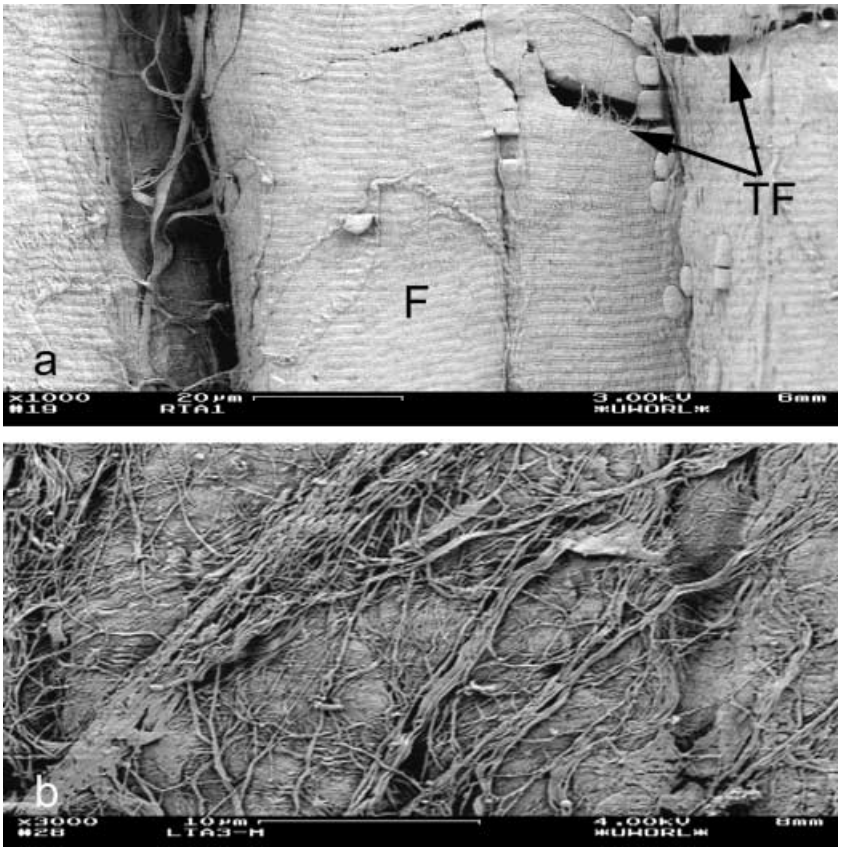

Fig. 4 SEM showing torn fibres in the distal muscle belly (a). TF, fibre tearing; F, muscle fibre. SEM showing 'gap' separations at the myotendinous junction in response to a minor muscle stretch (b).

maximum oxidant production (Brickson et al. 2001; St. Pierre Schneider et al. 2002). These early studies provided some rationale for our current thinking that neutrophil-derived oxidants lead to secondary damage following the initial mechanical insult.

Another important observation was that both the degree of muscle fibre damage and the leukocyte infiltration of stretch-injured muscle increase between 4 and 24 h post-injury (St. Pierre Schneider et al. 2002). Others have proposed that the initial mechanical insult following repetitive eccentric contractions is caused by sarcomere overstretching (Armstrong, 1990). A so-called 'secondary' injury attributable to inflammatory cell invasion may exacerbate the initial strength loss (Armstrong, 1990). Our results support the hypothesis that there is a secondary or collateral injury that is coincident with neutrophil invasion of the muscle (St. Pierre Schneider et al. 2002). These findings suggest that new treatment paradigms may be possible that are directed towards specific aspects of neutrophil function. In the next section, we present a plausible theory that neutrophil-derived oxidants are responsible for extending the damage that results from the initial stretch injury. Whether these strategies can be extended from animal models to humans is an important question to be explored.

Do neutrophils cause injury to skeletal muscle?

An intriguing topic is the role that the inflammatory process and its mediators play in the repair of injured muscle. Acute inflammation is regarded as the body's generalized protective response to tissue injury. Neutrophils, together with monocytes and macrophages, play a critical role in acute inflammation through removal of necrotic tissue or cellular debris and release of cytokines to modulate chemotaxis (Tidball, 1995). Neutrophils rapidly invade injured muscle tissue and provide several important functions that contribute to both inflammation and healing. Within 1 h following intense exercise, neutrophils invade skeletal muscle (Raj et al. 1998). Similarly, neutrophil counts are elevated within 2 h of return to muscle loading following a period of hindlimb suspension (Tidball et al. 1999). Studies show that these neutrophils contain and destroy damaged tissue or foreign matter primarily though phagocytosis. In addition, muscle proteins in damaged areas may also be degraded by proteases intrinsic to the muscle or by proteolytic systems introduced by infiltrating phagocytic cells. One consequence of neutrophil activation, however, is that these cells can undergo a respiratory burst and degranulation. Although their role in assisting with the removal of damaged tissue appears important, the oxidizing reactions associated with the respiratory burst may be responsible for collateral damage to healthy muscle during the early inflammatory period. Neutrophils can generate hypochlorous acid via an MPO-mediated reaction and superoxide ($O_2^{\bullet-}$) via NADPH oxidase that potentially can contribute further to muscle damage during modified use or injury. Superoxide is a mild oxidant that can rapidly be removed by reaction with other free radicals or by its conversion to hydrogen peroxide though superoxide dismutase (SOD). Hydrogen peroxide is a stronger oxidant that can be rapidly converted to more highly reactive oxidants including hydroxyl radicals and hypochlorous acid.

The majority of evidence for *in vivo* neutrophil-mediated myofibre damage comes from ischaemia-reperfusion (I/R) studies in which animals are rendered leukopenic using anti-neutrophil serum, anti-tumour agents, radiation or leukopak filters (Korthuis et al. 1988; Rubin Lescaudron et al. 1999). Several observations show that neutrophil-mediated damage during I/R is largely mediated by free radicals. Neutrophil depletion prior to I/R can attenuate histologically detectable damage by up to 40% in certain cases (Korthuis et al. 1988; Jolly et al. 1986; Kosonen et al. 2000). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) also contribute to skeletal muscle injury and necrosis associated with muscular dystrophies (Rando, 2001) as well as contraction-induced muscle injury (Reid, 1998).

Perhaps the most compelling study to argue that neutrophils cause skeletal muscle damage though an $O_2^{\bullet-}$-mediated mechanism involves a hindlimb suspension model (Nguyen & Tidball, 2003). In this investigation, mice deficient in NADPH oxidase (null mutation of $gp91^{phox}$) demonstrated a significant reduction in muscle fibre damage during reloading without changes in the concentrations of neutrophils and macrophages of the reloaded muscles. $Gp91^{phox}$ is the peptide subunit of NADPH oxidase, which is required for $O_2^{\bullet-}$ generation by neutrophils.

Several studies using our animal model suggest a distinct time course for oxidant production in stretch-injured skeletal muscle (Best et al. 1999; Brickson et al. 2001). Total oxidant levels (ROS, nitric oxide, and other

low-molecular-weight oxidants) of fresh tissue homogenates were estimated using a dichlorofluorescein (DCF) probe (LeBel & Bondy, 1990). A biphasic increase in total oxidant levels was noted in the stretch-injured leg (Brickson et al. 2001). In an attempt to clarify the role of individual oxidants further, we measured MPO, xanthine oxidase (XO) and SOD activities from muscle homogenates. MPO is expressed primarily by neutrophils (macrophages and monocytes produce small amounts) and is particularly important in determining the fate of hydrogen peroxide. MPO is commonly used as a marker of neutrophil accumulation within tissue and a marker of neutrophil activity when measured in plasma. MPO levels were elevated at 4 and 48 h, and XO activity was maximum at 24 h; in the latter case, however, both the control and the stretch-injured limbs showed similar changes (Brickson et al. 2001). Although neutrophil-generated $O_2^{\bullet-}$ plays a major role in muscle damage following I/R, superoxide itself is not likely to cause direct membrane damage in our model as SOD levels are maximum at 4 h post-injury when histologically discernible damage is not readily apparent (Brickson et al. 2001). These results should be interpreted with some caution given that the extent to which neutrophils promote muscle injury may also be affected in part by the history of muscle use as both training and modified activity can affect expression of SOD and nitric oxide synthase (Tidball et al. 1998; Nakao et al. 2000; Hollander et al. 2001).

To evaluate more completely the neutrophil's contribution to muscle damage, we have employed an anti-CD11b antibody (M1/70) to block the respiratory burst of infiltrating leukocytes (Brickson et al. 2003). Oxidants can affect muscle damage through a variety of mechanisms, including the promotion of chemotaxis, facilitating migration by up-regulating endothelial adhesion molecules, and enhancing neutrophil activation by up-regulating CD11b receptor density. Therefore, oxidants may play both a direct and an indirect role in muscle damage. Using the M1/70 approach, we have shown decreased oxidant production and CD11b receptor density in blood-borne neutrophils of animals treated with this antibody prior to creating a stretch injury to the tibialis anterior muscle (Brickson et al. 2003). In addition, this strategy reduced neutrophil infiltration into the muscle together with a three-fold reduction in myofibre damage at 24 h post-injury (Fig. 5a–c). Quantification of torn fibres at the muscle–tendon junction was limited to the muscle's medial edge and the haematoma was clearly restricted to the medial 20% of the cross-section, based on an average width of 1 cm (St. Pierre Schneider et al. 2002). These findings suggest blood-borne neutrophil oxidants as a potential target for treatment interventions aimed at limiting myofibre damage. Moreover, the CD11b receptor may be an effective target for minimizing oxidant production. The CD11b-dependent respiratory burst may be a great contributor to overall oxidant generation than sources

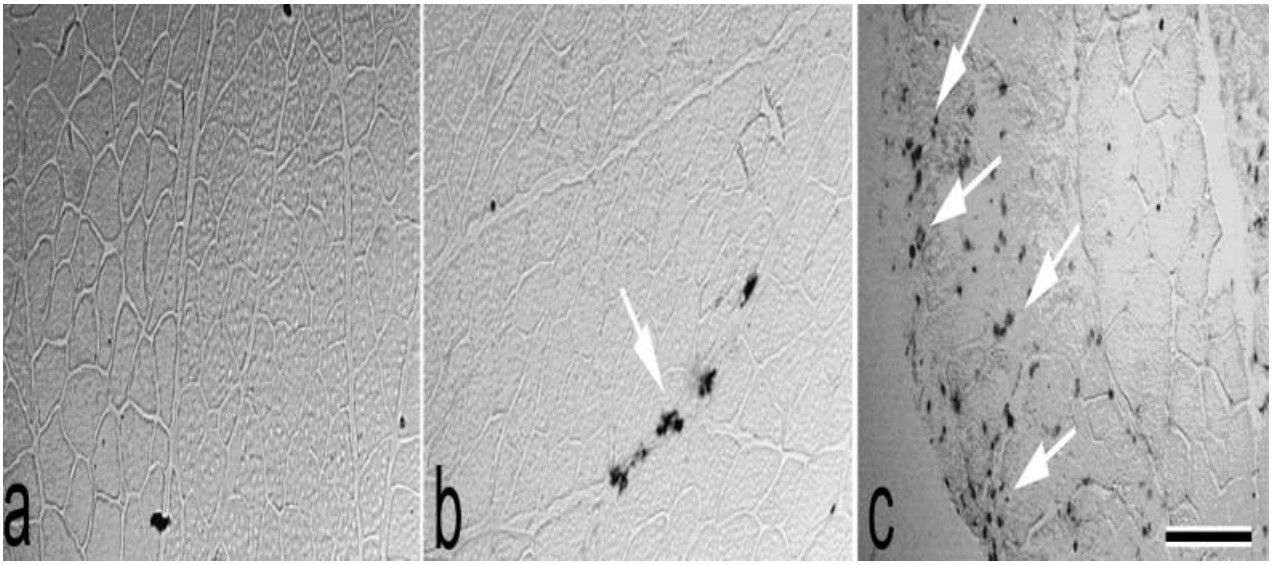

Fig. 5 IgG control (a) and M1/70-treated animals (b) at 24 h post-injury. Note the increased cellular infiltration (arrows) and fibre damage in the IgG-treated animal (c) compared with the M1/70-treated animal. Scale bar = 200 μm. Haematoxylin and eosin.

such as the cyclooxygenase and lipooxygenase pathways that are not blocked by M1/70. At least one common treatment, non-steroidal anti-inflammatories, interferes with the cyclooxygenase pathway. Our findings merit further investigation to determine if the approach of blocking CD11b affects recovery of muscle function at the same time that damage is attenuated. We postulate that the M1/70-induced decrease in CD11b receptor density was responsible for the significant reduction in Mab.198$^+$ and RPN3/57$^+$ cellular infiltration. Our findings are consistent with studies suggesting that perspectives for rational approaches to handle the development of skeletal muscle injury during neutrophil invasion be considered (Tidball et al. 1999; Frenette et al. 2003; McLoughlin et al. 2003; Nguyen & Tidball, 2003). Ultimately, re-evaluating the necessity of complete neutrophil function in the setting of muscle injury may be necessary. One possibility would be selectively to block NADPH oxidase without affecting leukocyte infiltration and determine its effects on extent of muscle damage. It may also be that our results are not generalizable to all forms of eccentric muscle damage. At least one study has shown that there was no difference in extent of tissue damage in the muscles of neutropenic and non-neutropenic mice subjected to a bout of 150 eccentric contractions (Lowe et al. 1995). Differences in exercise protocol and assay for muscle damage may help in part to explain these differences; nevertheless, these studies do suggest that some caution be exercised in assuming that neutrophils always cause tissue damage in models of injury and modified muscle activity.

Do neutrophils participate in muscle repair?

Although strong evidence is emerging that inflammatory cells cause muscle injury in a variety of models and species, it has also been suggested that they may function to promote muscle growth or regeneration that follows injury or disease (Tidball, 1995). The invasion of muscle by macrophages, particularly the ED2+ subpopulation, is essential in some circumstances for satellite cell activation and satellite cell-mediated muscle repair (Merly et al. 1999). A vital role for macrophages in muscle regeneration is plausible based on the observation that myogenesis is impaired in the absence of macrophage infiltration (Lescaudron et al. 1999). Macrophages can therefore promote muscle repair *in vitro*, although their role *in vivo* following muscle injury remains poorly understood.

How neutrophils contribute to muscle growth and repair following injury is unknown. The fact that muscle injury initiates a rapid invasion of the damaged tissue by neutrophils that can persist for days while muscle repair and regeneration occurs at the same time suggests that damage and repair may be mechanistically related. Only recently, however, have animal experiments been designed to begin to test this hypothesis and distinguish between the events of injury and repair. Mice treated by intraperitoneal injections of antisera to neutrophils and monocytes and injected with snake venom show a deficient regenerative response suggesting the importance of neutrophils for normal muscle repair (Teixeira et al. 2003). Another interesting observation from this study was that animals depleted of neutrophils and monocytes also showed more tissue debris in the injured muscles, raising the possibility at least that the impaired capacity to remove tissue debris by phagocytes could slow the regenerative process (Teixeira et al. 2003). Taken together, these observations with our findings on post-damage muscle leukocyte infiltration and collateral muscle damage suggest a possible role for neutrophils in muscle regeneration and repair. We have shown that neutrophils are the predominant cell infiltrating the muscle following injury at a time immediately preceding removal of necrotic tissue and activation of satellite cells (St. Pierre Schneider et al. 2002). As suggested by Teixeira et al. (2003), neutrophils could facilitate muscle regeneration by removing tissue debris from the injured area (Fielding et al. 1993; Tiidus, 1998) as well as by activation of satellite cells (Seale & Rudnicki, 2000). Could it also be possible that neutrophil-mediated damage is necessary for growth and repair?

Muscle damage and recovery seem to have a similar cellular mechanism, in that satellite cells become activated and fuse with the damaged muscle fibres. In response to an injury, satellite cells become activated and proliferate. Some of the satellite cells will re-establish a quiescent satellite cell pool through a process of self-renewal. Muscle satellite cell activation during growth and repair resembles embryonic myogenesis in many ways, including the *de novo* induction of myogenic regulatory factors such as MyoD and myogenin. Satellite cells will migrate to the damaged region and, depending on the severity of the injury, fuse to the existing myofibre or align and fuse to produce a new myofibre. In the regenerated myofibre, the newly fused satellite cell nuclei will initially be centralized but will later migrate to assume a more peripheral location.

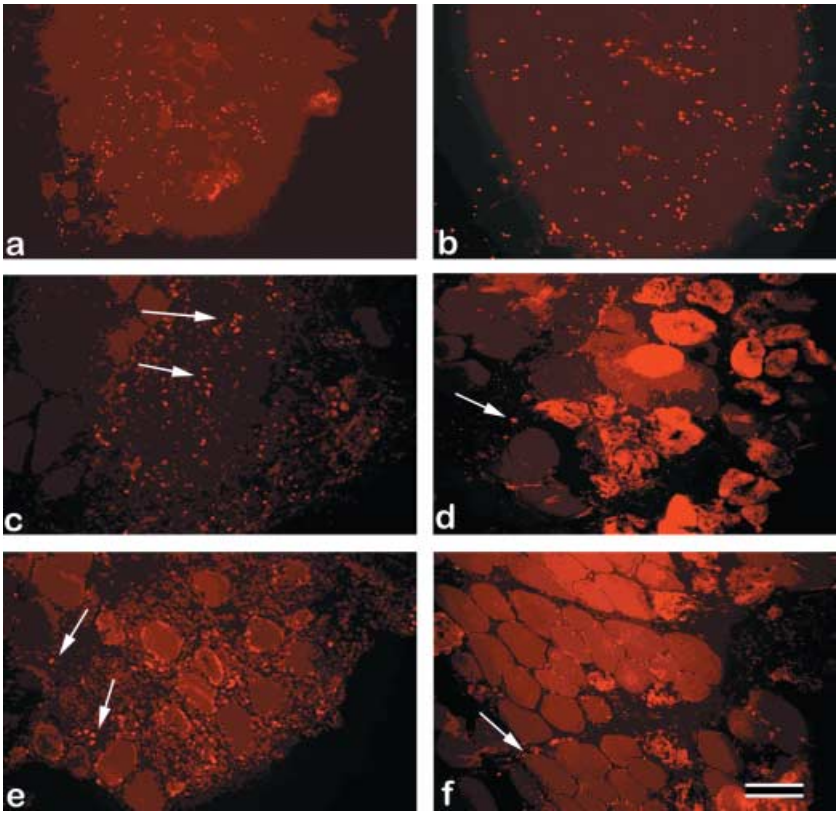

Fig. 6 BrdU labelling in the control (a) muscle was primarily localized in the area of injury. In the M1/70 muscles (b), however, BrdU labelling was widespread throughout the muscle. Numerous MyoD-positive nuclei (arrows) are in control muscles (c) in areas of damage. In M1/70 muscles (d), there are fewer labelled nuclei, suggesting that the early signals for muscle regeneration are down-regulated when neutrophil infiltration is blocked. IgG control muscles (e) contain numerous myogenin-poistive nuclei (arrows). In M1/70 muscles (f), there are fewer labelled nuclei, providing additional evidence that neutrophil infiltration is associated with myofibre regeneration. Scale bar = 200 μm.

To gain insight into the time course for muscle repair following stretch injury (unpublished studies) we have examined BrDU as a marker of general cell proliferation (Fig. 6a,b), coupled with expression of the muscle regulatory factors MyoD (Fig. 6c,d) and myogenin (Fig. 6e,f). We found that M1/70 treatment reduced fibre damage as shown previously (Brickson et al. 2003). Similarly, CD11b receptor blockade with M1/70 reduced total BrDU labelling in the area of maximum damage (fibre tearing) (Fig. 6a,b). The most dramatic effect of M1/70 treatment, however, was a marked reduction in both MyoD and myogenin labelling (Fig. 6c–f) at 72 h following injury. In the vicinity of damaged fibres, there was a reduction in the number of cells labelled for MyoD and myogenin compared with animals not treated with M1/70 (Fig. 6c–f). Alternatively, the sustained BrDU response at 72 h (Fig. 6b) may indicate the presence of proliferating non-myogenic cells such as fibroblasts that could favour muscle fibrosis and scarring. Assuming that the stimulus for muscle regeneration and repair is dependent on the magnitude of injury, we also analysed our data to control for differences in muscle damage and noted similar findings. In other words, controlling for the degree of damage still led to the observation that blocking the CD11b receptor and neutrophil infiltration led to a markedly decreased initial regenerative response. One intriguing hypothesis therefore is that reducing neutrophil infiltration results not only in reduced collateral damage but also in a reduction in the repair response. The mechanism for this attenuated repair response is unknown, but one explanation may be that phagocytosis and/or secondary damage through the respiratory burst are critical signals for muscle regeneration. Attenuation of these events could possibly decrease signalling for muscle repair. Further work is needed to confirm our preliminary observations that neutrophil infiltration of damaged muscle may impair myofibre regeneration (Fig. 7). Moreover, these observations come from a single time point (72 h post-injury) and the long-term consequences of this finding need to be determined. In other words, what are the consequences of blocking neutrophil infiltration on muscle repair at later time points? Once the mechanisms for this phenomenon have been discovered, therapeutic treatment strategies could be developed to manipulate selective events of muscle inflammation and repair in order to achieve optimal healing.

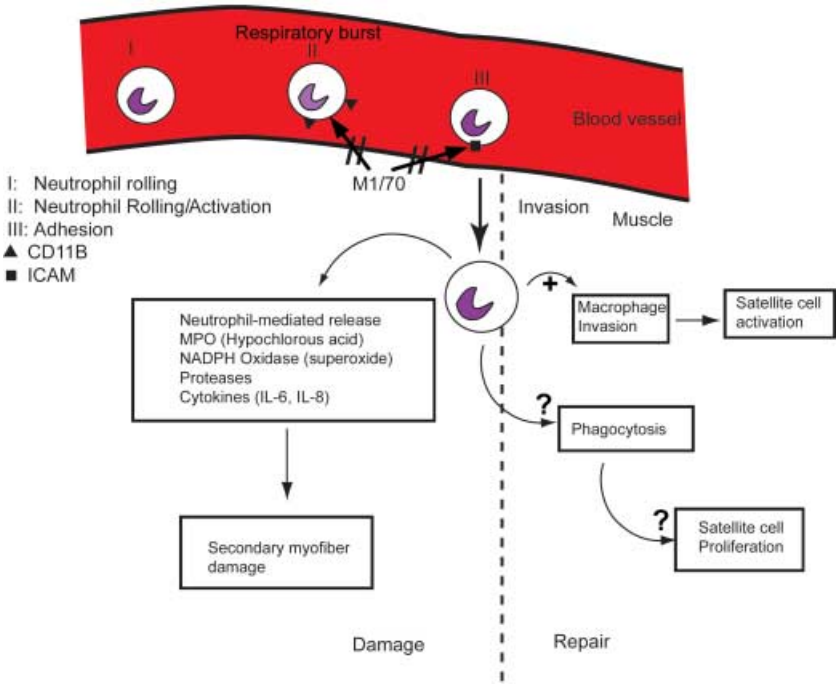

Fig. 7 Proposed schematic for the role of neutrophils in muscle injury and repair.

Muscle healing and fibrosis

A barrier to complete recovery following muscle injury may be scarring and fibrosis (Orchard & Best, 2002). In certain models of muscle trauma, healing is incomplete and scarring and fibrosis result (Hurme et al. 1991; Best et al. 2001; Sato et al. 2003). We have demonstrated formation of connective tissue scar containing Type III collagen within 3 days of injury (Best et al. 2001). There was increased mRNA expression of collagen III early in the post-injury period with little evidence for expression of muscle regeneration. Focal deposits of both Type I and Type III collagen were detected at the myotendinous junction by 7 days (Best et al. 2001). Our finding of early fibrosis at the same time as muscle repair is initiated agrees with the work of others suggesting that scar formation may occur at the expense of muscle regeneration (Hurme et al. 1991). It would seem therefore that strategies to reduce scar formation and, alternatively, enhance muscle regeneration would be appealing to improve recovery from muscle injury. Overproduction of TGF-β_1 has been associated with tissue fibrosis in injured skeletal muscle (Li et al. 2004). Moreover, the fibrosis and stiffness observed in dystrophic muscle probably involves the action of several inflammatory cytokines such as TGF-β_1 and TNF-α (Bernasconi et al. 1999). Injection of human recombinant decorin, a proteoglycan that inactivates TGF-β, reduces fibrosis and improves contractile properties following muscle laceration (Fukushima et al. 2001). Injection of Enbrel to block TNF-α reduces the expression of both TGF-β_1 and collagen 1 mRNA expression in the diaphragm of young *mdx* mice (Gosselin & Martinez, 2004). Taken together, these studies provide the first evidence that control of fibrosis and scarring may actually improve the muscle's regenerative response and recovery of function. Accordingly, the development of strategies to limit muscle scarring would seem plausible and worthy of investigation.

In order to gain insight into the potential cellular mechanisms responsible for muscle fibrosis and scarring (unpublished studies), we have quantified expression of vimentin, an intermediate filament that is expressed by myotubes and myoblasts early in development and shortly after injury. More importantly, the protein is not observed in regenerating or mature myofibres but is strongly expressed in fibrocytes of scar tissue (Bornemann & Schmalbruch, 1992; Li & Huard, 2002). Vimentin labelling in the control muscle is confined mainly to the region of injury whereas staining in the M1/70 samples was elevated thoughout the stretch-injured muscle (Fig. 8a,b). We confirmed these findings using a modified Masson's trichome stain to distinguish accurately between muscle and collagen. Our preliminary results (unpublished data) suggest that blocking neutrophil infiltration impairs the muscle's regenerative

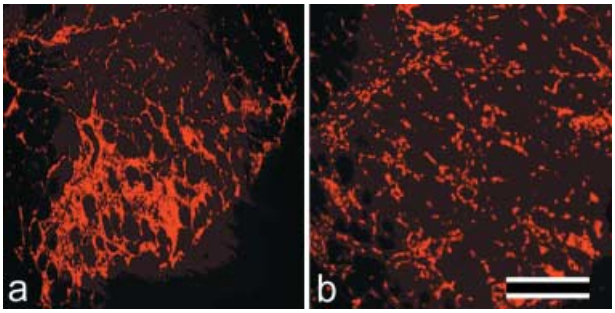

Fig. 8 Vimentin labelling in the control (a) muscle was primarily localized in the area of injury. In the M1/70 muscles (b), vimentin labelling was present throughout the stretch-injured muscle. Scale bar = 200 μm.

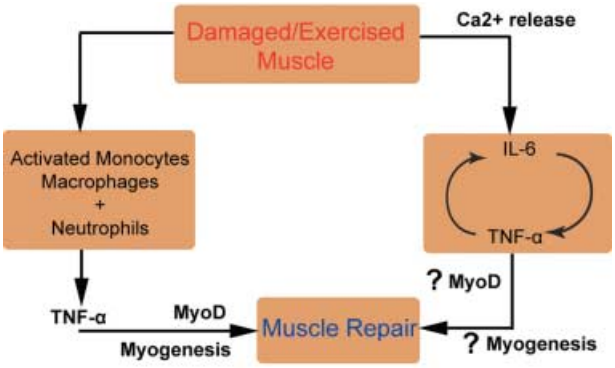

Fig. 9 Schematic diagram outlining the possible role for IL-6 in skeletal muscle injury and repair.

response while having little effect on non-muscle cellular proliferation. These findings argue that blocking neutrophil infiltration may not lead to optimal healing and in fact may favour a proliferative response that leads to muscle scarring and fibrosis. Future efforts will be directed towards understanding the signalling mechanisms that operate to modulate muscle regeneration though neutrophil infiltration. A more complete understanding of the role of neutrophils in regeneration and scarring and of the downstream signals may lead to an improved understanding and treatment of muscle damage.

IL-6 as a possible mediator of muscle healing

Compelling *in vitro* and *in vivo* evidence supports a role for both leukaemia inhibitory factor (LIF) and IL-6 in muscle regeneration. IL-6 is an intercellular ubiquitous cytokine traditionally associated with the control and co-ordination of immune responses. It is produced by many cell types, including stimulated monocytes and macrophages, fibroblasts, and vascular endothelial cells. Neutrophils and skeletal muscle cells are also a rich source of IL-6 under certain conditions.

Recent data suggest both a pro- and an anti-inflammatory role for IL-6 in muscle injury and inflammation. IL-6 is typically the first cytokine present in the circulation during exercise. Transgenic mice overexpressing IL-6 show marked muscle wasting together with enhanced activity of cathepsin B and L, suggesting that IL-6 may be involved in protein degradation and muscle damage (Tsukinaka et al. 1995). By contrast, IL-6 can induce proliferation of satellite cells, raising the possibility at least for a potential role for IL-6 in muscle regeneration (Cantini et al. 1995). Conversely, freeze injury results in increased IL-6 expression within the damaged muscle but IL-6-deficient mice demonstrate increased MyoD expression and recovery of isometric strength similar to that of wild-type mice (Warren et al. 2002). Therefore, it appears that IL-6 plays a role in muscle inflammation and repair with the exact details yet to be determined.

In order to help clarify the role of IL-6 in muscle injury and repair, we have begun to investigate the time course and localization of IL-6 expression following a stretch injury. At 24 h post-injury, damaged fibres stain positive for IL-6 (Fig. 9, unpublished studies). Double staining of the muscle with PAX-7 and RPN3/57 demonstrated that neither satellite cells nor invading inflammatory cells were co-expressing IL-6. We have confirmed similar findings using a crush injury in which IL-6 was expressed only by the damaged fibres and not the invading inflammatory cells. Treatment with M1/70 reduced both myofibre damage and IL-6 expression by these fibres. In an additional set of experiments, rabbits were pretreated 4 days before the experiments with Mechlorethamine to deplete the circulating neutrophils (Kishi et al. 1999). A stretch injury was created on Day 5 and analysis the following day showed decreased cellular infiltration and a four-fold decrease in torn fibres in the stretch-injured muscles of mechlorethamine-treated animals. Taken together, these experiments advance the hypothesis that neutrophils cause injury and suggest that the damaged myofibres are responsible for IL-6 production. Others have shown IL-6 synthesis within muscle following a series of eccentric contractions, despite no obvious signs of histological damage (Tomiya et al. 2004). The observation that similar increases of IL-6 mRNA in

both concentric and eccentric exercised muscle supports the idea that its production cannot be as closely related to muscle damage as first thought.

The exact role of IL-6 in stretch injury to skeletal muscle is a topic of ongoing investigations. The initial phase of muscle repair is characterized by necrosis of the damaged tissue together with activation of an inflammatory response. Tightly coupled to this necrotic phase is activation of a regenerative response that is highlighted by activation of satellite cells to proliferate, differentiate and fuse. We along with others have identified the presence of signalling factors such as IL-6 in damaged skeletal muscle, although its exact role remains unknown (Fig. 9). It is clear that the growth and metabolism of both healthy and injured muscle cells can be directly influenced by changes in the mechanical environment. Identification of the pathways through which signalling factors such as IL-6 influence muscle growth and repair will provide valuable insights into the interface between mechanical and chemical signalling of skeletal muscle.

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