IMPLICATIONS OF CROSS-TALK BETWEEN TUMOUR NECROSIS FACTOR AND INSULIN-LIKE GROWTH FACTOR-1 SIGNALLING IN SKELETAL MUSCLE

Miranda D Grounds,* Hannah G Radley,* Bijanka L Gebski,* Marie A Bogoyevitch† and Thea Shavlakadze*

*School of Anatomy and Human Biology, The University of Western Australia, Crawley, Western Australia and
†Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, Australia

SUMMARY

1. Inflammation, particularly the pro-inflammatory cytokine tumour necrosis factor (TNF), increases necrosis of skeletal muscle. Depletion of inflammatory cells, such as neutrophils, cromolyn blockade of mast cell degranulation or pharmacological blockade of TNF reduces necrosis of dystrophic myofibres in the mdx mouse model of the lethal childhood disease Duchenne muscular dystrophy (DMD).

2. Insulin-like growth factor-1 (IGF-1) is a very important cytokine for maintenance of skeletal muscle mass and the transgenic overexpression of IGF-1 within muscle cells reduces necrosis of dystrophic myofibres in mdx mice. Thus, IGF-1 usually has the opposite effect to TNF.

3. Activation of TNF signalling via the c-Jun N-terminal kinase (JNK) can inhibit IGF-1 signalling by phosphorylation and conformational changes in insulin receptor substrate (IRS)-1 downstream of the IGF-1 receptor. Such silencing of IGF-1 signalling in situations where inflammatory cytokines are elevated has many implications for skeletal muscle in vivo.

4. The basis for these interactions between TNF and IGF-1 is discussed with specific reference to clinical consequences for myofibre necrosis in DMD and also for the wasting (atrophy) of skeletal muscles that occurs in very old people and in cachexia associated with inflammatory disorders.

Key words: inflammation, insulin like growth factor 1, necrosis, skeletal muscle, tumour necrosis factor.

DUCHENNE MUSCULAR DYSTROPHY AND THERAPIES

Duchenne muscular dystrophy (DMD) is an inherited X-linked lethal childhood muscle disease caused by a defect in the gene for dystrophin, which affects young boys, causes extreme wasting and loss of function of skeletal muscles and leads to death usually by 20 years of age. Dystrophin is located beneath the sarcolemma and is part of a large dystrophin–dystroglycan complex that forms a critical link for force transmission between the contractile machinery of the muscle fibre and the extracellular matrix. Where dystrophin is defective or absent, the myofibre is fragile and the sarcolemma is readily damaged in response to exercise, leading to myofibre necrosis.1 Although it is widely considered that mechanical tears in the sarcolemma are the cause of the initial damage, other data indicate that changes in ion channels may be responsible for the initial influx of calcium that causes the damage;2 clearly, an accurate understanding of the basic mechanism will affect the targeting of potential therapeutic interventions. Although myofibre necrosis normally results in new muscle formation, in DMD (and, to a lesser extent, in the mdx mouse model of DMD) it appears that regeneration fails over time and the dystrophic muscle is progressively replaced by fatty and fibrous connective tissue.

Although the defective gene, dystrophin, was identified in 1987, there is still no effective treatment for DMD boys. Although cell or gene therapy to replace the defective dystrophin is the ideal scenario, the clinical application of such therapies is yet to become a reality.3,4 Meanwhile, many preclinical studies continue on the mdx mouse model of DMD.5

The existing treatment for DMD is administration of corticosteroids; these are broad-based anti-inflammatory drugs that decrease inflammatory cell populations in dystrophic muscle6 and increase myofibre mass, although the precise mechanism of action in DMD is not yet known and is under intense investigation.7,8 One disadvantage of steroids is that they are associated with severe adverse side-effects, such as weight gain and osteoporosis,9 and the response is variable between individual boys.10
Inflammatory response can directly damage myofibres in myopathic conditions, such as dystrophies or myositis, and recent data increasingly implicate inflammation, and specifically tumour necrosis factor (TNF), in myofibre necrosis.

Tumour necrosis factor is a major pro-inflammatory cytokine that is expressed by a wide range of inflammatory cells and by myoblasts, myotubes and damaged skeletal muscle. Tumour necrosis factor is also produced by adipose tissue that is often pronounced within the wasted skeletal muscles in DMD. In response to even minor myofibre injury, TNF is rapidly released from resident mast cells and also by neutrophils, which accumulate quickly at sites of tissue damage, and TNF is a potent chemokine that attracts further inflammatory cells to the injured site. The chemotactic role of TNF was demonstrated in normal mouse muscle, where administration of TNF resulted in the accumulation of neutrophils and macrophages in the absence of any tissue damage.

In support of the proposal that TNF and neutrophils exacerbate initial sarcolemmal damage and provoke necrosis of dystrophic myofibres, in vivo blockade of TNF, cromolyn prevention of degranulation of mast cells (which normally release high levels of TNF) or depletion of host neutrophils protects dystrophic mdx mouse muscle from necrosis. Two drugs that were used to block TNF activity in the mdx mouse model of DMD, namely infliximab (an antibody to TNF) and etanercept (soluble receptor to TNF) are in wide clinical use already to treat inflammatory disorders such as arthritis and Crohn’s disease. The high specificity of these anticytokine drugs, combined with their clinical success in other diseases and relatively few side-effects, suggests that they may be attractive alternatives to the existing use of corticosteroids to treat DMD. In the mdx mouse, long-term studies have further demonstrated that the mouse-specific cV1q antibody to TNF has equal efficacy to Remicade and Enbrel. It is noted that this cV1q blockade of TNF has no effect on the low levels of chronic myofibre damage in unexercised dystrophic muscle, in striking contrast with the marked protective effect on exercise-induced acute myofibre necrosis, raising the possibility of different roles for TNF (and other molecules) in these two situations of myopathology. The impact of exercise, as well as other factors such as age and gender that affect the severity of the pathology, should be taken into account when interpreting the expression profile of different molecules and the impact of drug interventions and other therapies in mdx mice.

That TNF protein is elevated locally in dystrophic muscles is supported by immunohistological studies showing increased staining for TNF associated with necrotic areas of dystrophic muscles of the mdx mouse (Fig. 1) and in biopsies from DMD patients, as well as Western blotting analysis using anti-TNF antibodies in mdx muscle extracts. It is noted that the issue of representative tissue from the small biopsy sample that can be taken from DMD muscles makes such measurements very difficult in humans. Few studies have quantified TNF in dystrophic muscles or blood. Although one study reported significantly higher plasma levels of TNF in dystrophic (DMD and Becker muscular dystrophy) patients than in age-matched control patients, another reported low levels of TNF levels in blood from DMD patients. It has proven difficult to detect elevation of TNF mRNA expression in skeletal muscles of adult non-exercised mdx mice (T Shavlakadze, unpubl. data, 2007) and it seems that TNF levels (protein and mRNA) have not been reported for dystrophic dogs.

Elevated TNF may exacerbate muscle damage through several pathways. One of the contributors to TNF induced muscle necrosis could be the inflammatory transcription factor nuclear factor...
**Fig. 2** Damaging effects of tumour necrosis factor (TNF) in skeletal muscle cells are mediated, in part, by interference with insulin-like growth factor (IGF)-1 receptor signalling. c-Jun N-terminal kinase (JNK1) has been identified as one of the signalling molecules that mediates such interference; JNK1 can associate with the IGF-1 docking protein insulin receptor substrate-1 (IRS)-1 and inhibit its activity, which would lead to downregulation of biological processes mediated by IGF-1 (i.e. stimulation of protein synthesis, inhibition of protein degradation and cell survival). Independent of the interaction with IGF-1 signalling, TNF may upregulate protein degradation, leading to muscle atrophy, and promotes myofibre necrosis. Nuclear factor-kB is one of the central players in both these processes. PI3-K, phosphatidylinositol 3-kinase; FOXO, forkhead box, subgroup O transcription factor.

We have undertaken intensive studies using transgenic mice that overexpress IGF-1 only within skeletal muscle. An important finding was the demonstration that the reduced pathology in mdx mice that overexpress the Class 1 IGF-1Ea isoform is likely due to reduced myofibre necrosis and this protective effect may relate to increased protein synthesis and decreased protein degradation. The signalling pathways of IGF-1 are highly complex, with effects on not only atrophy/hypertrophy via promotion of protein synthesis and inhibition of protein degradation, but also on apoptosis, myoblast proliferation and muscle differentiation.

To further complicate the situation, there are at least six isoforms of IGF-1 and the specific biological function of different isoforms of IGF-1 are not defined. The recent development of transgenic mice that overexpress these different isoforms (N Winn (EMBL, Italy), unpubl. data, 2007) will hopefully help clarify their relative importance in skeletal muscle. It is noted that although the Class 1 IGF-1Ea isoform clearly reduced the dystrophopathy of mdx mice, transgenic mdx mice that overexpress the fully processed 70 amino acid human IGF-1 within myofibres (Rskα-actin/hIGF-1 transgene) and have elevated IGF-1 in muscles and blood showed no improvement in muscle pathology. Whether this lack of effect reflects the different form of IGF-1 overexpressed within the muscle or is due to increased IGF-1 levels seen only in the blood as well as skeletal muscles of these mdx/hIGF-1 transgenic mice is unclear, but these contrasting findings emphasize the complexity of interpreting transgenic data.

**CROSS-TALK BETWEEN TNF AND IGF-1 SIGNALLING PATHWAYS VIA JNK**

One of the main mechanisms by which TNF causes myofibre atrophy and myofibre necrosis maybe signalling mediated by NF-κB. The blockade of NF-κB by pyrrolidine dithiocarbamate reduces skeletal muscle degeneration in mdx mice and it has recently been shown that heterozygous deletion of the p65 subunit of NF-κB is sufficient to decrease muscle necrosis in mdx mice.

Another possible mechanism for the damaging effects of TNF could be by activation of c-Jun N-terminal kinase (JNK). This is of special interest because activated JNK can inhibit the expression of insulin-like growth factor (IGF)-1 mRNA, as well as IGF-1 signalling, and such cross-talk between TNF and IGF-1 has many implications for muscular dystrophy and other conditions where inflammatory cytokines are elevated. A striking increase in phosphorylation of JNK1 has been reported in the diaphragm muscles of 7-week-old and 12-month-old mdx mice, whereas there was little increase in the limb muscles of 12-month-old mdx mice. Another study reported increased phosphorylation of JNK2, but not JNK1, in the limb muscle of 16-week-old non-exercised and exercised mdx mice. In marked contrast with the adverse effects of TNF on dystrophic muscle, increased levels of IGF-1 protect dystrophic muscle from necrosis and the roles of IGF-1 are discussed below.

**COMPLEX ROLES OF IGF-1 AND IMPORTANCE IN SKELETAL MUSCLE**

Insulin-like growth factor-1 plays a central role in myofibre hypertrophy and atrophy and this balance is of critical importance for muscle wasting in ageing (sarcopenia), in inflammatory disorders ( cachexia), denervation, disuse atrophy and metabolic syndrome.
Inhibition of the Ser307 residue leads to dissociation of IRS-1 from the IGF-1 receptor and inhibition of the tyrosine phosphorylation of IRS-1, which is required for the downstream signal transmission from the activated IGF-1 receptor. Use of the JNK inhibitors I-JNK and SP600125 has confirmed the effects of JNK as a negative regulator of IGF-1 signalling in C2C12 myoblasts, but these effects are yet to be tested in vivo. However, in cultured 3T3-L1 adipocytes, extracellular signal-regulated kinase (ERK) 1/2 rather than JNK seems to mediate IRS-1 phosphorylation at the Ser307 residue in response to TNF, because inhibition of ERK1/2 but not JNK1 was sufficient to abolish the Ser307 phosphorylation. In addition, the phosphorylation of IRS-1 on Ser307 takes place not only in response to TNF, but also following treatment with insulin and IGF-1, which represents a negative feedback loop responsible for insulin and IGF-1 resistance; this inhibition of IRS-1 by insulin and IGF-1 appears to be distinct from the signalling pathway activated by TNF.

The activation of a JNK1-mediated signal transduction cascade has been suggested to contribute to progression of the dystrophic mdx phenotype, independent of IRS-1 inhibition. Adenoviral expression of the JNK1 inhibitor JNK-interacting protein (JIP) 1 in skeletal myofibres of mdx mice that also lack MyoD protected them from degeneration and increased their cross-sectional area. That study suggested that the mechanism of JNK1 action in dystrophic muscle is due, at least in part, to serine phosphorylation and nuclear exclusion of the calcineurin-sensitive nuclear factor of activated T cells (NFAT) transcription factor. Data demonstrating the role of NFAT signalling in myofibre hypertrophy are controversial (for a review, see Shavlakadze and Grounds); however, in mdx muscle, upregulation of the calcineurin/NFAT pathway is protective against muscle degeneration. Furthermore, although deflazacort (a steroid used to treat DMD boys) did not alter JNK1 activity itself, it increased activity of the calcineurin phosphatase and upregulated NFAT-dependent gene expression, which, in turn, negates JNK1 inhibition. Taken together, these results suggest that further evaluation of JNK inhibitors, including JNK inhibitory peptides, and JNK ATP competitive inhibitors as new treatments for muscular dystrophy (with potential clinical application to DMD) should be considered.

Cross-talk between IGF-1 and TNF is further complicated by a report that IGF-1 can inhibit TNF signalling involved in protein catabolism, as shown in human colonic adenocarcinoma cells where pretreatment with IGF-1 reduced TNF-mediated nuclear localization of NF-κB. It was suggested that muscle-specific elevation of IGF-1 would also intercept TNF signalling and reduce the loss of muscle mass (cachexia) in inflammatory conditions; moreover, it has been demonstrated recently that inhibition of NF-κB signalling protects against denervation-induced muscle atrophy. Experiments are required to test the in vivo possibility that IGF-1 may play an inhibitory role in inflammatory mediated wasting of skeletal muscle.

**BEYOND DYSTROPHY: CLINICAL IMPLICATIONS FOR AGING AND OTHER MUSCLE CONDITIONS**

Maintenance of skeletal muscle mass is governed by a complexity of signalling interactions and age-related muscle weakness and loss of muscle function (sarcopenia) presents many serious problems. Human studies show that, in the elderly, systemic low-grade inflammation associated with increased blood levels of TNF and interleukin-6 can contribute to loss of muscle mass and strength. Cytokines are responsible for muscle protein degradation in more severe cases of inflammation, such as cancer cachexia, sepsis and AIDS. Muscle wasting produced by TNF is associated with induction of oxidative stress, which is considered to be a major contributor to age-related sarcopenia. It has been suggested that the effects of TNF on muscle atrophy may also be mediated, in part, via interference with IGF-1 signalling and inhibition of the anabolic signalling cascade downstream of the IGF-1 receptor, which would lead to decreased protein synthesis and upregulation of atrophy-related genes. Thus, attempts to minimize muscle wasting in various clinical conditions have focused on both anti-inflammatory drugs to block TNF action and the development of strategies to deliver IGF-1 to skeletal myofibre. Clarification of interactions between these two opposing pathways presents the possibility of new therapeutic targets and should provide valuable insight into molecular events determining the severity of muscular dystrophy and other muscle disorders.

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