Implications of cross-talk between TNF and IGF-1 signalling in skeletal muscle

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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 transgenic over-expression 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 *via* the phosphorylation and conformational change in IRS1 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.

Duchenne muscular dystrophy and therapies

Duchenne muscular dystrophy (DMD) is an inherited X-linked lethal childhood muscle disease, due to 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] While 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. While 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. While 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 pre-clinical 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 muscle[6] and increase myofibre mass, although the precise mechanism of action in DMD is not yet known and is under intensive 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]

While waiting for gene correction therapies to hopefully become a clinical reality, attention has turned to drug and nutritional interventions that target inflammation, fibrosis, necrosis, muscle mass and reactive oxygen species, designed to reduce the severity of the dystropathology (reviewed in Tidball & Wehling-Hendricks,[11] Radley *et al.*[12]). The best combinations of such drugs[13] alone or with dietary interventions[14] for long-term use in DMD remains to be determined.[12]

Role of TNF in muscular dystrophy

There is increasing evidence that inflammation contributes to the necrosis of dystrophic myofibres.[15,16] When myofibre breakdown and necrosis occurs, inflammation and associated cytokines are essential for removal of necrotic tissue and for formation of new skeletal muscle. Inflammatory cells and a range of cytokines influence myoblast activation, migration, proliferation, differentiation and fusion. Strong evidence that inflammatory cells can contribute to necrosis of healthy muscle cells comes from studies investigating the role of neutrophils, macrophages and oxidative damage *in vitro*[17-20] and *in vivo*.[21-23] It has similarly been proposed that an excessive inflammatory response can directly damage myofibres in myopathic conditions such as dystrophies or myositis[15,24] and recent data increasingly implicate inflammation and specifically tumour necrosis factor (TNF) in myofibre necrosis.

TNF is a major pro-inflammatory cytokine that is

expressed by a wide range of inflammatory cells and by myoblasts, myotubes and damaged skeletal muscle.[25,26] TNF is also produced by adipose tissue[27,28] 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 that accumulate quickly at sites of tissue damage[16,29] 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.[30]

In support of the proposal that TNF and neutrophils exacerbate initial sarcolemmal damage and provoke necrosis of dystrophic myofibres, *in vivo* blockade of TNF,[31-33] cromolyn prevention of degranulation of mast cells (that normally release high levels of TNF),[29] or depletion of host neutrophils[32] protects dystrophic mdx mouse muscle from necrosis. Two drugs that were used to block TNF activity in the mdx mouse model of DMD, Remicade® (antibody to TNF, also known as Infliximab) and Enbrel® (soluble receptor to TNF, also known as Etanercept) are in wide clinical use already to treat inflammatory disorders such as arthritis and Crohn's disease. The high specificity of these anti-cytokine 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 cVIq antibody to TNF has equal efficacy to Remicade® and Enbrel®.[34] It is noted that this cV1q blockade of TNF has no effect on the low levels of chronic myofibre damage in un-exercised dystrophic muscle, in striking contrast to 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.[34] The impact of exercise and also 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 also 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 that show increased staining for TNF associated with necrotic areas of dystrophic muscles of the mdx mouse (Figure 1)[35] and biopsies from DMD patients[26] and also with Western blotting analysis using anti-TNF antibodies in mdx muscle extracts.[36] 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 for humans. Few studies have quantitated TNF in dystrophic muscles or blood. While one study reported significantly higher plasma levels of TNF in dystrophic (DMD and Becker MD) patients than in age-matched control patients,[37] another reported low levels of TNF levels in blood from DMD patients.[38] It has proved difficult to detect elevation of TNF mRNA expression in skeletal muscles of adult non-exercised mdx mice (Shavlakadze – unpublished data) and it seems that TNF levels (protein and mRNA) have not been reported for dystrophic dogs.

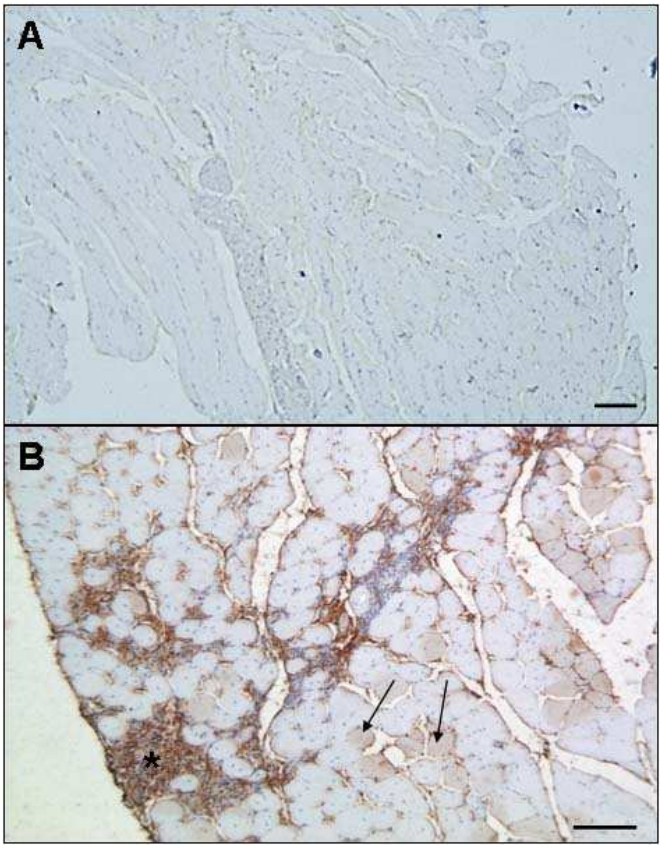

Figure 1. *Immunohistochemical staining of TNF in transverse sectioned dystrophic (mdx) and non-dystrophic (C57Bl/10) mouse muscle (TNF stains brown). Non-dystrophic muscle (A) shows no specific staining for TNF, scale bar represents 100μm. Dystrophic muscle (B) shows TNF staining on the plasma membrane of muscle fibres and also in areas of focal necrosis (*), with faint staining in some apparently intact myofibres (arrows), scale bar represents 100μm.*

Elevated TNF may exacerbate muscle damage through several pathways.[39] One of the contributors to TNF induced muscle necrosis could be the inflammatory transcription factor nuclear factor-kappaB (NF-κB), since NF-κB is activated in limb muscles[40] and the diaphragm[40,41] of mdx mice and also in muscle samples from DMD patients.[42] The blockade of NF-κB by pyrrolidine dithiocarbamate reduces skeletal muscle degeneration in mdx mice[36] 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.[40]

Another possible mechanism for the damaging effects of TNF could be by activation of Jun N-terminal kinase (JNK). This is of special interest since activated JNK can inhibit expression of IGF-1 mRNA and also IGF-1

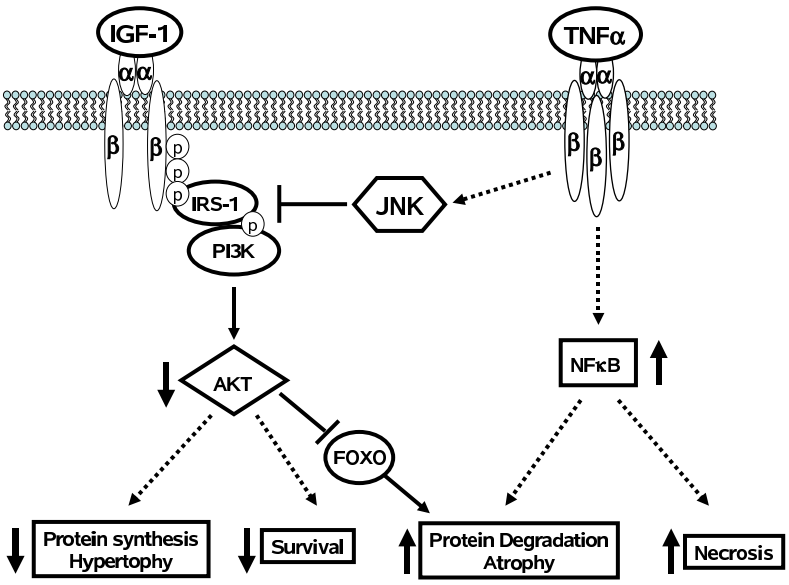

Figure 2. *The damaging effects of TNF in skeletal muscle cells are partially mediated by interference with IGF-1 receptor signalling. JNK1 has been identified as one of the signalling molecules that mediates such interference. JNK1 can associate with the IGF-1 docking protein IRS-1 and inhibit its activity, which would lead towards down-regulation of biological processes mediated by IGF-1, i.e. stimulation of protein synthesis, inhibition of protein degradation and cell survival. Independently from interaction with IGF-1 signalling, TNF may up-regulate protein degradation, leading to muscle atrophy and promotes myofibre necrosis. NF-*KB *is one of the central players in both these processes*

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 weeks[43] and 12 month old mdx mice,[44] whereas there was little increase in the limb muscles of 12 month old mdx.[44] Another study reported increased phosphorylation of JNK2, however not JNK1 in the limb muscle of 16 weeks old non-exercised and exercised mdx mice.[45] In marked contrast to the adverse effects of TNF on dystrophic muscle, increased levels of insulin-like growth factor-1 (IGF-1) protect dystrophic muscle from necrosis[46] and the roles of IGF-1 are discussed below.

Complex roles of IGF-1 and importance in skeletal muscle

IGF-1 plays a central role in myofibre hypertrophy and atrophy[47,48] and this balance is of critical importance for muscle wasting in ageing (sarcopenia), in inflammatory disorders (cachexia), denervation, disuse atrophy and also in the metabolic syndrome.[48] We have carried out intensive studies using transgenic mice that over-express IGF-1 only within skeletal muscle.[46,49-51] An important finding was the demonstration that the reduced pathology in mdx mice that over-express the Class 1 IGF-1Ea isoform is likely due to reduced myofibre necrosis[46] 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,[48] but also on apoptosis, myoblast proliferation and muscle differentiation.

To further complicate the situation there are at least 6 isoforms of IGF-1 and the specific biological function of different isoforms of IGF-1 are not defined.[52,53] The recent development of transgenic mice that over-express these different isoforms (N Winn, EMBL, Italy, unpublished data) will hopefully help clarify their relative importance in skeletal muscle. It is noted that, while the Class 1 IGF-1Ea isoform clearly reduced the dystropathology of mdx mice,[46, 54] transgenic mdx mice that over-express the fully processed 70aa long 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.[51] Whether this lack of effect reflects the different form of IGF-1 over-expressed 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,[51] but these contrasting findings emphasise the complexity of interpreting transgenic data.[53]

Cross-talk between TNF and IGF-1 signalling pathways *via* JNK

One of the main mechanism by which TNF causes myofibre atrophy and myofibre necrosis maybe signalling mediated by NF-κB[36,55] (Figure 2). However, the deleterious effects of TNF on skeletal muscle may also be

due to interference with IGF-1 signalling.

TNF may inhibit IGF-1 dependent events by down-regulation of IGF-1 synthesis[56] and inhibition of signalling pathways downstream of the IGF-1 receptor.[57-59] Activation of JNK appears to play a role in both of these processes and this molecule is the focus of the following discussion. Evidence for an inhibitory action of TNF on IGF-1 synthesis is based on *in vivo* and tissue culture experiments.[56] Mice injected with a non-lethal dose of lipopolysaccharide (known to stimulate inflammation) show increased TNF protein in blood and increased expression of TNF mRNA in skeletal muscle, with decreased blood levels of IGF-1 and decreased IGF-1 mRNA expression in skeletal muscle.[56] More direct proof that TNF regulates IGF-1 expression is provided by a tissue culture experiment where direct addition of TNF to C2C12 myoblasts and differentiated myotubes decreased IGF-1 mRNA.[56] JNK activation has been suggested to mediate TNF induced inhibition of IGF-1 synthesis in C2C12 myoblasts, since in these cultured skeletal muscle cells the JNK inhibitor, SP600125, blocked the TNF induced decrease in IGF-1 mRNA expression.[56]

Interference of TNF with IGF-1 signalling may occur *via* inhibition of the IGF-1 receptor docking proteins Insulin Receptor Substrate IRS-1 and IRS-2,[57-59] leading to down-regulation of signalling molecules further downstream of the IGF-1 receptor, that are involved in regulation of the protein balance and cell survival (Figure 2). Tissue culture studies suggest that in C2C12 myoblasts treated with TNF, JNK associates with IRS-1 and phosphorylates serine 307 (Ser307).[60] Phosphorylation 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.[61] 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,[60] but these effects are yet to be tested *in vivo*. However, in cultured 3T3-L1 adipocytes, ERK1/2 rather than JNK seems to mediate IRS-1 phosphorylation at the Ser307 residue in response to TNF,[62] since 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;[62] 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 from IRS-1 inhibition. Adenoviral expression of the JNK1 inhibitor JIP1 in skeletal myofibres of mdx mice that also lack MyoD protected them from degeneration and increased their cross sectional area.[44] This study suggested that the mechanism of JNK1 action in dystrophic muscle is at least partly due to serine phosphorylation and nuclear exclusion of the calcineurin sensitive NFAT transcription factor. Data demonstrating the role of NFAT signalling in myofibre hypertophy are controversial (reviewed in Shavlakadze &, Grounds[48]), however in mdx muscle upregulation of the calcineurin/NFAT pathway is protective against muscle degeneration.[63] Furthermore, while deflazacort (a steroid used to treat DMD boys) did not alter JNK1 activity itself, it increased activity of the calcineurin phosphatase and up-regulated NFAT-dependent gene expression which in turn negates JNK1 inhibition.[64] Taken together, these results suggest that further evaluation of JNK inhibitors including JNK inhibitory peptides and JNK ATP competitive inhibitors[65,66] 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 pre-treatment with IGF-1 reduced TNF mediated nuclear localization of NF-κB.[67] 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 recently been demonstrated that inhibition of NF-κB signalling protects against denervation induced muscle atrophy.[68] 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 ageing and other muscle conditions

Maintenance of skeletal muscle mass is governed by a complexity of signalling interactions[48] and age-related muscle weakness and loss of muscle function (sarcopenia) presents many serious problems.[69,70] Human studies show that in the elderly, systemic low-grade inflammation associated with increased blood levels of TNF and IL-6 can contribute to loss of muscle mass and strength.[71,72] Cytokines are responsible for muscle protein degradation in more severe cases of inflammation, such as cancer cachexia, sepsis and AIDS.[55] Muscle wasting produced by TNF is associated with induction of oxidative stress,[55] 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[47] and inhibition of the anabolic signalling cascade downstream of the IGF-1 receptor, that would lead to decreased protein synthesis and up-regulation 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 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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