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Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment

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Abstract

Tumour necrosis factor (TNF) is a potent inflammatory cytokine that appears to exacerbate damage of dystrophic muscle *in vivo*. The monoclonal murine specific antibody cV1q that specifically neutralises murine TNF demonstrated significant anti-inflammatory effects in dystrophic mdx mice. cV1q administration protected dystrophic skeletal myofibres against necrosis in both young and adult mdx mice and in adult mdx mice subjected to 48 h voluntary wheel exercise. Long-term studies (up to 90 days) in voluntarily exercised mdx mice showed beneficial effects of cV1q treatment with reduced histological evidence of myofibre damage and a striking decrease in serum creatine kinase levels. However, in the absence of exercise long-term cV1q treatment did not reduce necrosis or background pathology in mdx mice. An additional measure of well-being in the cV1q treated mice was that they ran significantly more than control mdx mice.

Keywords: DMD; Mdx mouse; TNF; Myofibre necrosis; Voluntary exercise

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disorder, affecting approximately 1/3500 male births [1,2]. Complete absence or impaired function of the skeletal muscle protein dystrophin leaves dystrophic myofibres susceptible to damage during mechanical contraction [3,4]. Consequently this initial damage progresses to myofibre necrosis. Repeated cycles of necrosis ultimately result in replacement of myofibres by fat and fibrotic connective tissue and loss of muscle function [2,5]. It is hypothesised that the initial myofibre damage is exacerbated by the endogenous inflammatory response [6–9] and that inflammatory cells and cytokines further damage the sarcolemma resulting in myofibre necrosis rather than the repair of minor membrane lesions. There is strong evidence to suggest that inflammatory cells and cytokines play a role in skeletal muscle damage (reviewed in [8]) and dystrophic muscle tissue has a considerably different gene expression pattern compared to non-dystrophic muscle with up-regulation of multiple genes involved in both the inflammatory response and muscle regeneration [10]. Blockade or depletion of resident T cells [11], neutrophils [9], macrophages [12,13] and mast cells [14,15] in mdx mice *in vivo* reduces the severity of dystropathology.

These cells all produce tumour necrosis factor (TNF) that is a potent pro-inflammatory cytokine that induces chemokine expression and upregulates adhesion protein expression on endothelial cells, resulting in cell infiltration to sites of inflammation [16,17]. TNF was previously referred to as TNFα, however as discussed in a recent review [18] the renaming of TNFβ as lymphotoxin (LTα and LTβ) leaves TNFα an orphan term and thus the appropriate term for use is now TNF. TNF is elevated in both DMD and mdx mouse muscles [19–21]. Antibody blockade of TNF with the human/mouse chimeric antibody infliximab (Remicade®) in young mdx mice, results in a striking protective effect on dystrophic myofibres and suppresses the early acute phase of myofibre necrosis [7]. A similar

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protective effect in young dystrophic muscle was demonstrated with etanercept (Enbrel® – a soluble TNF receptor) [9]. These results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle. In addition, both infliximab and etanercept treatment prevented the inflammatory response normally seen at 5 days in whole muscle autografts in non-dystrophic C57BL/10 mice, confirming the efficacy of these drugs in mice [22].

The monoclonal antibody infliximab was generated to block human TNF [23], and is currently very effectively used in the treatment of Crohn's disease and rheumatoid arthritis [24]. There is controversy regarding the cross-species binding of infliximab based on *in vitro* tests, yet at least four papers report anti-inflammatory effects *in vivo* in mice [7], rats [25,26] and pigs [27]. To increase the efficacy of TNF blockade in mice and to avoid potential problems of immune response to the human constant domain sequences of infliximab, a rat monoclonal antibody specific for mouse TNF [28] and chimerized using mouse kappa light chain and mouse 1gG2a heavy chain constant domain sequences (cV1q) was identified [29]. The present paper tests the effectiveness of the cV1q (mouse-specific anti-TNF) antibody in both dystrophic and non-dystrophic mice, in both short and long-term (up to 90 days) studies combined with voluntary exercise. The use of the species specific antibody is considered important for long-term studies to minimise immune problems.

The mdx mouse, an animal model for DMD [30], undergoes a spontaneous onset of acute necrosis and subsequent regeneration in limb and paraspinal muscles around 3 weeks of age [7,31]. The high level of muscle necrosis between 21 and 28 days provides an excellent model to study therapeutic interventions designed to prevent or reduced muscle necrosis, as a reduction in dystropatholgy is easily observed [7,15,32,33]. Myofibre necrosis markedly decreases and stabilises by 6 weeks of age [34,35]. The low level of dystropathology in adult mice can be made significantly worse by exercise that increases myofibre necrosis and reduces muscle strength [14,36,37] enabling potential therapeutic interventions to be evaluated in adult mdx mice [15,38–41].

2. Experimental overview

The cV1q antibody was tested in four *in vivo* models of inflammation. (1) Whole muscle autografts in adult non-dystrophic C57BL/10ScSn mice (the non-dystrophic parental strain for mdx), (2) young dystrophic mdx mice (male and female littermates), (3) adult dystrophic (mdx) mice subjected to 48 h voluntary exercise and (4) long-term (up to 90 days) cV1q treatment in both exercised and unexercised adult dystrophic (mdx) mice. Voluntary wheel running has several advantages compared to forced treadmill exercise; mice are able to run at night when they are normally active [42,43] which avoids the stress associated with exercising mice during the day when they are normally inactive, also voluntary exercise is less stressful to the animal than forced high intensity exercise [44,45]. We also hypothesise that the amount of voluntary exercise undertaken by an individual mouse may reflect the overall health of the mouse and serve as an additional measure of well-being to test drug interventions. The effects of cV1q are compared with infliximab and etanercept for three of the experimental models [7,9,22].

3. Material and methods

Mice. Experiments were carried out using dystrophic mdx litters (male and female littermates), dystrophic female mdx (C57BL/10ScSn$^{mdx/mdx}$) mice and non-dystrophic female C57BL/10ScSn mice (Table 1). All mice were obtained from the Animal Resources Centre (ARC) Murdoch, Western Australia, housed under a 12 h day–night cycle and allowed access to food and water *ad libitum*. Mice were treated in strict accordance with the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics.

cV1q treatment. Intra-peritoneal (IP) injections of cV1q and the isotype-matched, negative control antibody (cVAM) both provided by Centocor were given at a concentration of 20 μg/g/mouse/week (Table 1). For the whole muscle autografts (experiment 1) cV1q was routinely injected 24 h prior to surgery (unless otherwise stated). In young mdx mice (experiments 2 and 4) injections began at 19 days of age. In the 48 h voluntarily exercised adult mice (experiment 3) a single injection was given 24 h prior to voluntary exercise.

Whole muscle autograft surgery. Whole muscle autografts were used as an *in vivo* bio-assay to assess the anti-inflammatory properties of cV1q in non-dystrophic mice. Six-weeks-old female C57BL/10 mice were anaesthetised using 2% (v/v) Rodia Halothane. The extensor digitorum longus (EDL) muscle with both tendons was removed form the anatomical bed and transplanted onto the surface of the tibialis anterior (TA) muscle, the EDL tendons were sutured to the TA, the skin closed and wound left to heal, as described in [22,46,47]. It is well-documented that this

Table 1
Animal treatment summary

Experiment	Strain	Age	Sex	cV1q treatment
(1) Whole muscle autografts	C57BL/10	6 weeks	Female	1 day prior to surgery*
(2) Necrosis onset in young mdx litters	Mdx	d19–28	Mix	d19–d28 (weekly)
(3) 48 h voluntary exercise	Mdx	6 weeks	Female	1 day prior to exercise
(4) Long-term cV1q treatment	Mdx	d19–90	Female	d19–d90 (weekly)

* Indicates that cV1q injections were usually given 1 day prior to whole muscle graft surgery; however some injections occurred at 1 week prior to surgery and some at 1 week prior to surgery plus again at 5 days after surgery.