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Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice

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Abstract

Necrosis of skeletal muscle fibres in the lethal childhood myopathy Duchenne Muscular Dystrophy results from deficiency of the cell membrane associated protein, dystrophin. We test the hypothesis in dystrophin-deficient mice, that the initial sarcolemmal breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells, specifically neutrophils, and that cytokines, specifically Tumour Necrosis Factor alpha (TNFα), contribute to myofibre necrosis. Antibody depletion of host neutrophils resulted in a delayed and significantly reduced amount of skeletal muscle breakdown in young dystrophic mdx mice. A more striking and prolonged protective effect was seen after pharmacological blockade of TNFα bioactivity using Etanercept. The extent of exercise induced myofibre necrosis in adult mdx mice after voluntarily wheel exercise was also reduced after Etanercept administration. These data show a clear role for neutrophils and TNFα in necrosis of dystrophic mdx muscle in vivo. Etanercept is a highly specific anti-inflammatory drug, widely used clinically, and potential application to muscular dystrophies is suggested by this reduced breakdown of mdx skeletal muscle.

Keywords: TNFα; Etanercept; Neutrophils; Muscular dystrophy; Myofibre necrosis; Therapy

1. Introduction

Multinucleated skeletal muscle fibres are formed during development and in response to injury by the proliferation, differentiation and fusion of mononucleated precursor cells called myoblasts [1]. In newborn mice, myoblast proliferation and fusion decreases rapidly after birth and by 2 weeks most of the myoblasts are quiescent [2,3]. In dystrophic mdx mice, a model for the X-linked lethal human muscle disease Duchenne Muscular Dystrophy (DMD), there is a sudden increase in muscle damage and intensive regeneration at 3 weeks of age [2]. After this initial bout of necrosis in the young mdx mice muscle breakdown markedly decreases and stabilises at a relatively low level [4]. However, this sustained low level of myofibre necrosis can be significantly increased by exercise [5,6] and adult mdx mice subjected to exercise have been used to test potential therapeutic interventions designed to either reduce or prevent muscle necrosis [7–11] The susceptibility of dystrophic myofibres to damage is caused by defective or absent dystrophin, a protein that normally links the internal structure of the myofibre, through the cell membrane to the extracellular matrix [12,13].

It is hypothesised that inflammatory cells play a role in enhancing skeletal muscle damage and necrosis. Strong evidence that inflammatory cells contribute to necrosis of healthy muscle cells comes from

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investigations into the role of neutrophils, macrophages and oxidative damage in vitro [14–16] and in vivo [17–19], and it has been proposed that an excessive inflammatory response can directly damage myofibres in myopathic conditions such as dystrophies or myositis [20]. DNA microarray studies of mdx muscle found that 30% of all differentially expressed genes were associated with inflammation [20,21] and histological analysis of mdx muscle shows increased inflammatory cells, with the majority being macrophages and lymphocytes [22] as well as mast cells [23,24]. The role of inflammatory cells in muscle damage is the subject of an excellent recent review [25].

TNFα is a key cytokine that stimulates the inflammatory cell response. A role for TNFα in muscular dystrophy was proposed by Spencer and colleagues [26] and tested in TNFα null mdx mice [26,27], although the anticipated marked improvement of dystropathology did not result. In contrast, pharmacological blockade of TNFα activity with the neutralizing antibody Infliximab in mdx mice [28] was effective and clearly delayed and reduced the breakdown of dystrophic mdx muscle. Infliximab (also known as Remicade®) is a chimeric monoclonal antibody composed of murine variable and human constant regions [29]. Another highly successful clinical intervention that renders TNFα biologically inactive is the soluble TNF-receptor Etanercept (also known as Enbrel®), which is a dimeric fusion protein composed of an extracellular ligand-binding portion of the human (p75) TNF-receptor (TNFR) linked to the Fc portion of human IgG. Etanercept inhibits binding of both TNFα and TNFβ (lymphotoxin alpha (LTα)) to cell surface TNFRs [30]. Clinically, both Etanercept and Infliximab are highly effective at reducing symptoms of inflammatory diseases such as Rheumatoid Arthritis and Crohn's Disease and are being extended to other inflammatory conditions [30–32]. Etanercept has been reported to reduce the expression of both TGFβ and collagen mRNA in dystrophic mdx mice possibly leading to reduced fibrosis in muscles [33].

TNFα is expressed by both myoblasts and myotubes [34] and is greatly increased within damaged myofibres and myopathic skeletal muscle [35]. TNFα is also produced by adipose tissue [36], which is of interest because fat cells can replace much of the muscle tissue in DMD. In response to myofibre injury, TNFα is rapidly released from resident mast cells [11,37] and also released by neutrophils that accumulate quickly at sites of tissue damage [25]. While TNFα plays a central role in increasing the inflammatory cascade it seems likely that factors produced by neutrophils may be largely responsible for causing the additional damage to the myofibres [25]. Both neutrophils and TNFα play an early crucial role in myofibre necrosis and therefore both of these aspects are targeted in the present study.

The efficacy of antibody depletion of host neutrophils is compared with the effects of Etanercept (that blockades TNFα function) to reduce myofibre necrosis and protect dystrophic myofibres from the acute onset of damage in the mdx mouse model. A further comparison is made with previous data for Infliximab (Remicade®) treated mdx mice [28]. The acute onset of dystropathology at 3 weeks of age in the mdx mouse is a very sensitive assay for assessing the impact of interventions designed to reduce the necrosis of dystrophic myofibres [28,39]. There are differences in the onset of dystropathology of various muscles, but the tibialis anterior (TA) initially shows more necrosis than quadriceps and diaphragm [11,39]. Therefore, the TA muscle of mdx mice was analysed in the present study during the crucial week from 21 to 28 days of age, to assess the effects on myofibre necrosis of host neutrophil depletion and systemic Etanercept blockade of TNFα. A second model was used to test the ability of Etanercept to reduce myofibre necrosis in adult mdx mice subjected to voluntary wheel running for 48 h: in these mdx mice, the extent of myonecrosis in the quadriceps muscle (that is most damaged by voluntary exercise [11,40]) was quantified and serum creatine kinase (CK) levels measured as an additional marker of myofibre damage [9].

2. Materials and methods

2.1. Animals

All experiments were carried out using female mdx ($C57BL/10ScSn^{mdx/mdx}$) mice. Young mice were aged between 17 and 28 days and the exercised adult mice were 6–7 weeks of age: mice were specific pathogen free and obtained from the Animal Resources Centre Murdoch, Western Australia. Mice were housed in individual cages under a 12 h day–night cycle and allowed access to food and water ad libitum. Mice were treated according to 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 Committee.

2.2. Neutrophil depletion

Age matched mdx mice were injected intraperitoneally every second day starting at day 19, with 500 μg of cytotoxic rat anti-mouse granulocyte (GR-1) monoclonal antibody (RB6-8C5) to specifically (and continuously) deplete neutrophils [41]. This treatment typically depletes granulocytes similarly in both blood and spleens of female mdx mice ~80–95% for 5 days using daily intraperitoneal injections of 300 mg purified RB6-8C5. Granulocyte numbers in blood and spleens rapidly decline within 12 h of treatment, remain low for 5 days and then rapidly return to pre-treatment