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Immunomodulation of TGF-beta1 in mdx mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: Implications for antifibrotic therapy

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Abstract

Irreversible connective tissue proliferation in muscle is a pathological hallmark of Duchenne muscular dystrophy (DMD), a genetic degenerative muscle disease due to lack of the sarcolemmal protein dystrophin. Focal release of transforming growth factor-beta1 (TGF-β1) is involved in fibrosis development. Murine muscular dystrophy (mdx) is genetically homologous to DMD and histopathological alterations comparable to those in DMD muscles occur in diaphragm of older mdx mice. To investigate the early development of fibrosis and TGF-β1 involvement, we assessed diaphragms in 6–36-week-old mdx and C57/BL6 (control) mice for fibrosis, and used real-time PCR and ELISA to determine TGF-β1 expression. Significantly greater fibrosis and TGF-β1 expression were found in mdx from the 6th week. Mice treated with neutralizing antibody against TGF-β1 had lower levels of TGF-β1 protein, reduced fibrosis, unchanged muscles fiber degeneration/regeneration, but increased inflammatory cells (CD4+lymphocytes). These data demonstrate early and progressive fibrosis in mdx diaphragm accompanied by TGF-β1 upregulation. Reduction of TGF-β1 appears promising as a therapeutic approach to muscle fibrosis, but further studies are required to evaluate long term effects of TGF-β1 immunomodulation on the immune system.

Keywords: Muscular dystrophy; mdx animal model; Muscle fibrosis; Transforming growth factor-β1; Fibrogenic cytokine; Immunomodulation

1. Introduction

Abnormal connective tissue proliferation following myofiber degeneration is a major pathologic feature of Duchenne muscular dystrophy (DMD), a severe genetic myopathy due to a lack of the sarcolemmal protein dystrophin, and clinically characterized by progressive and irreversible degeneration of muscle tissue (Sanes, 1994; Engel et al., 1994). The proliferation of muscle extracellular matrix, characterized by deposition of fibronectin and type I and III collagens in the endomysium and perimysium of muscle tissue (Foidart et al., 1981; Stephens et al., 1982; Duance et al., 1980), leads to irreversible derangement of muscle organization, by impeding the regeneration of muscle fibers and hindering nutritional support, particularly in advanced stages when fibers are physically isolated from their blood supply (Engel et al., 1994; Duance et al., 1980). Since this fibrotic proliferation is likely to be a major obstacle to the efficacy of therapies for muscular dystrophies, early interventions to prevent it will probably be necessary as part of an effective treatment protocol.

Abnormal connective tissue proliferation also occurs in liver cirrhosis, glomerulonephritis, idiopathic lung fibrosis and systemic sclerosis. In these conditions, focal release of fibrogenic cytokines, particularly transforming growth factor-beta1 (TGF-β1) is a key element in promoting fibroblast proliferation and collagen synthesis (Kovacs, 1991). TGF-β1 is a multifunctional cytokine with roles in inflammation, immunomodulation, and wound healing, as well as fibrosis (Border and Noble, 1994). A significant correlation between

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fibrosis and TGF-β1 expression in Duchenne and Becker muscular dystrophies has been reported, supporting a role for this cytokine in the development of muscle fibrosis, and suggesting it as target for antifibrotic therapies (Bernasconi et al., 1995; Yamazaki et al., 1994).

Murine X-linked muscular dystrophy (mdx) and DMD are genetically homologous conditions characterized by a complete absence of dystrophin due to mutations in the dystrophin gene. The mdx mouse is a useful animal model for DMD (Hoffman and Dressman, 2001). Although the limb muscles of adult mdx mice show much less weakness, muscle degeneration and fibrosis than DMD boys, the diaphragm exhibits severe degeneration and functional impairment similar to that seen in DMD (Stedman et al., 1991). In fact, the extracellular matrix of mdx diaphragm muscle doubles between 60 and 120 days, and is increased by 8-fold at 240 days (Louboutin et al., 1993). However, the extent of fibrosis and TGF-β1 expression in early stages of the disease is poorly documented. Hartel et al. (2001) found increased TGF-β1 expression by ELISA in the diaphragm at 12-week-old mdx mice, and suggested its involvement in fibrosis; while Gosselin et al. (2004) reported early overexpression of TGF-β1 transcripts in mdx diaphragm, as well as inhibition of type I collagen mRNA, after decorin administration, but did not assess muscle fibrosis or TGF-β1 protein expression.

Studies on animal models of other diseases characterized by fibrosis have shown that reduction of TGF-β1 levels in affected tissues (skin, lung, thyroid and kidney) can limit fibrotic development (McCormick et al., 1999; Chen et al., 2002; Ziyadeh et al., 2000). To investigate whether the mdx mouse diaphragm exhibits a pattern of early fibrosis and early TGF-β1 overexpression, similar to that in human dystrophic muscle, and to provide indications as to the utility of early antifibrotic therapy, we studied mdx and C57/BL6 mice from 6 to 36 weeks of age, assessing fibrosis by morphometry (De Luca et al., 2005) and using ELISA and real-time PCR to determine TGF-β1 expression. Since we found significantly greater fibrosis and TGF-β1 from 6 weeks, we performed additional experiments to test the effect of limiting TGF-β1 activity by administering monoclonal antibody against the cytokine. In view of indications that TGF-β1 can have both pro- or antiinflammatory effects (Wahl, 1992), that inflammation can worsen muscle degeneration (Chen et al., 2000; Porter et al., 2002) and may interfere with muscle regeneration (Engel and Arahata, 1986; Spencer et al., 2001), we also investigated effects of anti-TGF-β1 antibody administration on inflammation, regeneration and degeneration in mdx diaphragm muscle.

2. Materials and methods

2.1. Animals

All experiments were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conform to European Community Directive 86/609/EEC. Twenty-five mdx mice (Jackson Laboratories, Bar Harbor, ME, USA) and 25 C57/BL6 mice (Charles River, Calco, Italy) were used to investigate the development of muscle fibrosis and expression of TGF-β1 between the ages of 6 and 36 weeks. Eighteen additional mdx mice were used to investigate the effects of anti-TGF-β1 treatment on muscle fibrosis, TGF-β1 expression, degeneration, regeneration and inflammation at 12 weeks. The animals were sacrificed by cervical dislocation; the diaphragms were removed rapidly, folded, rolled up and frozen in isopentane pre-cooled in liquid nitrogen.

2.2. Anti-TGF-β1 treatment

Eleven mdx mice of age 6 weeks were injected intraperitoneally with 300 μg of anti-TGF-β1 (1D11.16.8, HB 9849 ATCC, Manassas, VA, USA) on alternate days to age 12 weeks. Seven mdx mice of the same age were injected intraperitoneally at the same times with 300 μg normal mouse IgG (Pierce, Rockford, IL, USA). During anti-TGF-β1 treatment, the mdx mice did not shown abnormal behaviour, or differences in gross vital functions compared to untreated mdx mice and IgG-treated mice.

2.3. Morphometric analysis

Morphometric analysis was carried out on C57/BL6 mice as controls and also on untreated mdx mice and on those treated with anti-TGF-β1 or normal IgG. Serial cryostat cross-sections (6–8 μm thick) of rolled diaphragm were stained with Meyer's haematoxylin and eosin or Masson trichrome. The extent of endomysial and total (endomysial plus perimysial) connective tissue was determined on haematoxylin and eosin-stained sections (with Masson trichrome-stained sections used to check morphology) at ×20 magnification using the NIH Image software. At least three randomly selected fields from each section were analyzed. The area of connective tissue as a percentage of total muscle in each field was calculated, and the mean percentage for each group of animals calculated (De Luca et al., 2005).

2.4. Immunostaining

Immunohistochemical analysis was carried out on untreated mdx mice and on those treated with anti-TGF-β1 or normal IgG. Serial 6–8-μm-thick cryostat cross-sections of rolled diaphragm were cut, collected onto polylysine-coated slides, and fixed with ice-cold acetone for 1 min. Subsequent steps were performed in a humid chamber at room temperature. The sections were treated with peroxidase block solution (DAKO, Glostrup, Denmark) for 5 min followed by protein block solution