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Skeletal muscle-specific overexpression of IGFBP-2 promotes a slower muscle phenotype in healthy but not dystrophic *mdx* mice and does not affect the dystrophic pathology

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ABSTRACT

Objective: The insulin-like growth factor binding proteins (IGFBPs) are thought to modulate cell size and homeostasis via IGF-I-dependent and -independent pathways. There is a considerable dearth of information regarding the function of IGFBPs in skeletal muscle, particularly their role in the pathophysiology of Duchenne muscular dystrophy (DMD). In this study we tested the hypothesis that intramuscular IGFBP-2 overexpression would ameliorate the pathology in *mdx* dystrophic mice.
Design: 4 week old male C57Bl/10 and *mdx* mice received a single intramuscular injection of AAV6-empty or AAV6-IGFBP-2 vector into the tibialis anterior muscle. At 8 weeks post-injection the effect of IGFBP-2 overexpression on the structure and function of the injected muscle was assessed.
Results: AAV6-mediated IGFBP-2 overexpression in the tibialis anterior (TA) muscles of 4-week-old C57BL/10 and *mdx* mice reduced the mass of injected muscle after 8 weeks, inducing a slower muscle phenotype in C57BL/10 but not *mdx* mice. Analysis of inflammatory and fibrotic gene expression revealed no changes between control and IGFBP-2 injected muscles in dystrophic (*mdx*) mice.
Conclusions: Together these results indicate that the IGFBP-2-induced promotion of a slower muscle phenotype is impaired in muscles of dystrophin-deficient *mdx* mice, which contributes to the inability of IGFBP-2 to ameliorate the dystrophic pathology. The findings implicate the dystrophin-glycoprotein complex (DGC) in the signaling required for this adaptation.

1. Introduction

Muscle wasting and weakness are major symptoms of many neuromuscular disorders, including Duchenne muscular dystrophy (DMD). Although considerable efforts are being directed to the development of gene therapies for DMD, these techniques are far from perfected. In the interim, it is essential that alternative therapies are developed with research directed to preserving muscle mass, enhancing muscle regeneration, and promoting muscle growth. Therapeutic strategies are needed to ameliorate the dystrophic pathology and enhance patient quality of life. A successful pharmacological approach may enable patients to survive and thus take advantage of gene therapies when they eventually become available.

Modulating the insulin-like growth factor-I (IGF-I) signaling pathway is a potential therapeutic intervention for DMD. Signaling via the IGF-I axis promotes muscle regeneration by supporting the proliferation and differentiation of satellite cells [1], and chronic upregulation of IGF-I either by direct infusion, viral over-expression or in transgenic mice, induces muscle hypertrophy and attenuates age-related muscle wasting or sarcopenia [2–7]. In *mdx* mice, skeletal muscle specific transgenic upregulation of IGF-I improves force production and tissue pathology [8] and IGF-I administration protects muscles from contraction-induced injury [6,9]. However, the short half-life and acute side-effects, such as hypoglycemia, associated with IGF-I delivery limit its clinical application.

The biological actions of IGF-I in vivo are strongly modulated by a family of six IGF-binding proteins (IGFBPs) that bind ~99% of IGF-I in the circulation [10]. This large reservoir of bound, biologically

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inactive IGF-I has a prolonged half-life and is protected from degradation [11–13]. The primary roles of IGFBPs include transport of IGF-I to target tissues, modulation of IGF-I action, and prevention of hypoglycemia. Although it has been postulated that IGFBPs inhibit IGF-I actions in vitro and in vivo by preventing IGF-I binding to cell surface receptors [14,15], there is an increasing awareness that IGFBPs can have both enhancing and inhibiting effects on IGFs as well as IGF-independent effects [16,17].

While the role of IGFBPs in other tissues such as bone is relatively well understood [18–21], there is a dearth of information regarding the role of IGFBPs in skeletal muscle, particularly in the pathophysiology of muscular dystrophy. Of the IGFBP family, only IGFBP-5 has been directly investigated in muscular dystrophy, with increased expression in DMD fibroblasts correlating with impaired DMD myoblast growth in vitro [22]. Gene microarray studies performed on muscle biopsies from DMD patients revealed IGF-I was upregulated highly within dystrophic muscles, a response accompanied by transcriptional upregulation of IGFBPs (IGFBP-2, −4, and −6) [23]. Interestingly, IGFBP-2 was expressed at both higher and lower levels than controls dependent on the ages examined, suggesting that the roles of the IGFBPs in dystrophy are complex and warrant investigation [24]. A similar upregulation of IGF-I was reported in skeletal muscles of mdx mice, except that IGFPB-1 and −2 were downregulated and IGFBP-4 upregulated [25].

While each member of the IGFBP family is likely to have a unique function [26], there is strong evidence that IGFBP-2 plays a critical role in modulating muscle growth with multiple studies showing that increased IGFBP-2 expression has a negative effect on growth [21,27–32]. Increased serum IGFBP-2 concentrations have been correlated with low body weight in male mice [18]. Furthermore, mice with systemic over-expression of IGFBP-2 are significantly smaller than their non-transgenic littermates and have smaller myofibers [27,29,33]. Additionally, IGFBP-2 gene expression decreases with the progression of differentiation in C2C12 cells in vitro, indicating a potential role in muscle regeneration [34,35]. It has long been recognised that small calibre muscle fibers are more resistant to dystrophic pathology in *mdx* mice and DMD patients [36,37] and so the ability to reduce muscle mass has implications for protective effects. We therefore tested the hypothesis that intramuscular overexpression of IGFBP-2 ameliorates the dystrophic pathology in *mdx* mice.

2. Materials and methods

2.1. Animals

Male C57BL/10 and C57BL/10ScSn *mdx* (*mdx*) mice were obtained from the Animal Resources Centre (ARC) WA, Australia. All experimental protocols were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health & Medical Research Council (Australia).

2.2. Viral vector preparation and in vivo analyses

The cDNA encoding the murine IGFBP-2 coding region flanked by *SacII* and *HindIII* recognition sites was purchased from GenScript (New Jersey, USA), which was subsequently cloned into the *SacII*/*HindIII* locus of an rAAV6 expression plasmid containing the cytomegalovirus (CMV) immediate early enhancer and promoter, flanking inverted terminal repeats, and an SV40 polyA sequence [38]. All rAAV6 viral preparations were prepared as described previously, using a two-plasmid co-transfection method into HEK-293 cells followed by heparin-affinity purification [39]. Vector titer was evaluated via qPCR using the following primers and probe: Probe- 6FAM-ACTCATCAATGTATCTTATCATG-MGBNFQ; Forward primer- TTTTCACTGCATTCTAGTTGTGGTT; Reverse primer- CATGCTCTAGTCGAGGTCGAGAT.

rAAV6 ($1x10^{10}$ vg) was injected into the right tibialis anterior (TA) muscles of 3–4 week old male C57BL/10 and *mdx* mice ($n = 11$/genotype/virus). At 8 weeks post-injection mice were either killed and the right and left TA muscles were dissected and either snap-frozen in liquid nitrogen for biochemical analysis ($n = 3$/genotype/virus), or mice were anesthetized for assessment of contractile properties ($n = 8$/genotype/virus).

2.3. Assessment of contractile properties of skeletal muscle and tissue collection

Mice (n = 8/genotype/virus) were anesthetized with sodium pentobarbitone (Nembutal; 60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) via *i.p.* injection. The methods for assessment of the contractile properties of the mouse TA muscle in situ have been described in detail previously [40]. At the conclusion of the contractile measurements in situ, the TA muscle was carefully excised, blotted on filter paper and weighed on an analytical balance, then frozen in thawing isopentane for later histological examination.

2.4. Real-time qPCR

To examine the expression of the IGFBP genes, total RNA was isolated from gastrocnemius muscle samples of 3 week, 10 week and 30 week old C57BL/10 and *mdx* mice ($n = 4$–6/age/genotype) using the All-in-one purification kit (Norgen Biotek Corporation, Thorold, Canada) according to the manufacturer's guidelines. RNA quality and concentration were determined by a spectrophotometer at 260 nm and 280 nm and two ng reverse-transcribed RNA were used in reaction mixtures containing each 5 μM forward and reverse primers and Light Cycler 480 SYBR Green I Master (Roche, Mannheim, Germany) according to the manufacturer's instructions. Expression of IGFBP1–6 was assessed with the primer sequences described in Table 1 and were normalized to ActB. Templates were amplified after 5 min at 95 °C by 40 cycles of the following program: 15 s at 95 °C for denaturation, 30 s for annealing, and 30 s at 72 °C for extension. On the basis of the melting curve analysis from the PCR products, a high-temperature fluorescence acquisition point was determined and included in the amplification cycle program for 10 s. For all assays, a standard curve, generated by amplifying serial dilutions of specific PCR products, was analyzed using the LightCycler 2.0 (Roche, Mannheim, Germany) to determine assay efficiency and correlation coefficient.

For all remaining gene expression analyses, TA muscles ($n = 3$/genotype/virus) were excised at the conclusion of treatment and total RNA extracted using a commercially available kit, according to the manufacturer's instructions (RNeasy Fibrous tissue Mini Kit; Qiagen, Hilden, Germany). RNA quality and concentration were determined by a spectrophotometer at 260 nm and 280 nm and subsequently

Table 1
Primer sequences used for real-time qPCR analyses.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
IGFBP1	TTCAGCTCCCAGCATGAAG	GGGCTCTCAGAAAGCTCATC
IGFBP2	GCGGGTACCTGTGAAAAGAG	CCTCAGAGTGGTCGTCATCA
IGFBP3	GCAGCCTAAGCACCTACCTC	TCCTCCTCGGACTCACTGAT
IGFBP4	AAGATCGTGGGGACACCTC	GTGGGTACGGCTCTGTGAG
IGFBP5	GGCGAGCAAACCAAGATAGA	AGGTCTCTTCAGCCATCTCG
IGFBP6	GGGCTCTATGTGCCAAACTG	CCTGCGAGGAACGACACT
B-actin	TGACAGGATGCAGAAGGAGA	CGCTCAGGAGGAGCAATG
F4/80	CATCAGCCATGTGGGTACAG	CATCACTGCCTCCACTAGCA
TGFβ1	TGAGTGGCTGTCTTTTGACG	TCTCTGTGGAGCTGAAGCAA
Col1a1	CACCCTCAAGAGCCTGAGTC	GTTCGGGCTGATGTACCAGT
Col2a1	GCCAAGACCTGAAACTCTGC	GCCATAGCTGAAGTGGAAGC
Col3a1	ACCAAAAGGTGATGCTGGAC	GACCTCGTGCTCCAGTTAGC
Col4a1	AAAGGGAGAAAGAGGCTTGC	CCTTTGTACCGTTGCATCCT
Col5a1	GGTCCCTGACACACCTCAGT	TGCTCCTCAGGAACCTCTGT
Col6a1	CCCCATTGGACCTAAAGGAT	TCTCCCACTTCACCCTCATC