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Insulin-like growth factor I slows the rate of denervation induced skeletal muscle atrophy

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Abstract

Loss of the nerve supply to skeletal muscle results in a relentless loss of muscle mass (atrophy) over time. The ability of insulin-like growth factor-1 to reduce atrophy resulting from denervation was examined after transection of the sciatic nerve in transgenic MLC/mIGF-1 mice that over-express mIGF-1 specifically in differentiated myofibres. The cross sectional area (CSA) of all types of myofibres and specifically type IIB myofibres was measured in tibialis anterior muscles from transgenic and wild-type mice at 28 days after denervation. There was a marked myofibre atrophy (~60%) in the muscles of wild-type mice over this time with increased numbers of myofibres with small CSA. In the muscles of MLC/mIGF-1 mice, over-expression of mIGF-1 reduced the rate of denervation induced myofibre atrophy by ~30% and preserved myofibres with larger CSA, compared to wild-type muscles. It is proposed that the protective effect of mIGF-1 on denervated myofibres might be due to reduced protein breakdown.

Keywords: Denervation; IGF-1; Transgenic mouse; Atrophy; Skeletal muscle

1. Introduction

Denervation results in the loss of muscle mass and contractile force with concomitant alterations in morphology, ultrastructure, pattern of gene expression and biochemistry in myofibres and the surrounding tissue [1–10]. Atrophy of muscle fibres is most severe during the early (acute) stage of denervation and the fast type myofibres are more susceptible to denervation induced atrophy, than are slow type myofibres [1,2,5,7]. Denervation may also play a central role in the age-related myofibre atrophy associated with progressive loss of myofibre mass and strength (reviewed in [11]). Apart from the atrophy of myofibres, long-term denervated muscle gradually loses its restorative capacities [6,12]. Due to the progressive nature of atrophic processes secondary to denervation, the functional recovery of a muscle following nerve repair appears inversely related to the denervation time. Clinically, the rehabilitation strategies for patients with nerve lesions are aimed at restoration of the size of myofibres, contractile force and fatigue resistance of the denervated muscles and promotion of reinnervation [13].

Insulin-like growth factor-1 (IGF-1) is a potent regulator of myogenesis [14], skeletal muscle metabolism [15] and plays a central role in myofibre atrophy and hypertrophy (reviewed in [16]). In situ and in vivo models with skeletal muscle specific post-mitotic over-expression of IGF-1 have demonstrated a hypertrophic action of IGF-1 on myofibres [17,18]. Skeletal muscle specific over-expression of the MLC/mIGF-1 transgene in vivo promotes the functional hypertrophy of young skeletal muscles, rescues old skeletal muscles from age-related atrophy [18] and protects dystrophic mdx myofibres from breakdown [19,20].

Based on these previous studies which confirm a hypertrophic action of mIGF-1 and also demonstrate a protective effect on both age-related myofibre atrophy and breakdown of dystrophic myofibres, we hypothesized that over-expression of mIGF-1 would slow the rate of atrophy in skeletal muscle following short-term (28 days) denervation.

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2. Materials and methods

2.1. Animals

The experiments were conducted on transgenic MLC/mIGF-1 mice and their background strain wild-type FVB/N mice. A colony of these mice was established at the Animal Resources Centre in Perth, WA [21]. Presence of the MLC/mIGF-1 transgene was screened by polymerase chain reaction using tail digests as described previously [21]. Mice were allocated into four experimental groups: wild type intact, wild type denervated, MLC/mIGF-1 intact and MLC/mIGF-1 denervated, caged separately and maintained at 12 h light/dark regime with free access to standard chow and drinking water. Experiments were conducted in strict accordance with the guidelines of the University of Western Australia Animal Ethics Committee and the National Health and Medical Research Council, Canberra, Australia.

2.2. Denervation surgery, tissue sampling and processing

For denervation surgery, 8-week-old mice were anesthetized with a gaseous mixture of 1.5% Rodia Halothane (Merial, Australia), N_2O and O_2. The left sciatic nerve was exposed at the thigh level, by splitting the gluteal muscle and severed without disturbing the vasculature. To avoid re-innervation an approximately 5–7 mm nerve segment was excised and the skin was closed and sutured with 6-0 silk-braded suture (Johnson & Johnson, Sydney, Australia). The contra-lateral leg was left un-operated to allow normal activity of the animals. The mice were sacrificed by cervical dislocation at 28 days post surgery. For every mouse, close examination of tissues in the thigh region under surgical microscope confirmed that the severed sciatic nerve was still retracted, there was no evidence of any nerve re-growth or sprouting and reinnervation had not occurred. The tibialis anterior (TA) muscles were excised from denervated legs, dissected transversally in the mid belly region, mounted on tragacanth gum and frozen in liquid nitrogen quenched isopentane. The TA muscles from the age-matched (12-week-old) intact animals were also sampled and used as a control. Transverse sections (8 μm) of the mid belly region of the frozen muscles were cut on a cryostat (Leica CM3050), collected onto silane (3-aminopropyl-triethoxysilane) coated glass slides, air-dried and stored at −20 °C until used for histochemical or immunocytochemical staining. One section from each frozen block was stained with hematoxylin and eosin (H&E) for overall morphological assessment.

2.3. Histochemistry with nicotinamide adenine dinucleotide—nitro-blue tetrazolium (NADH)-TR

Histochemistry with NADH-TR was used to differentiate fast and slow type myofibres [22]. Muscle sections were incubated in NADH-TR (1:1) solution for 30 min at 37 °C, followed by 3 washes in distilled deionized water (DDW) and three exchanges of increasing (30, 60, 90%) and decreasing acetone solutions (30 s each). The slides were rinsed with DDW and mounted with polyvinyl acetate (PVA) aqueous mounting media.

2.4. Double immunocytochemistry against type IIB myosin and laminin

The sections were double labeled with primary mouse anti-type IIB myosin polyclonal antibody (clone 10F5) [23] (undiluted supernatant) and rabbit anti-laminin polyclonal antibody (#L9393, Sigma, Australia) (dilution 1:200). Binding of endogenous mouse immunoglobulins was blocked using Vector M.O.M. immunodetection kit (Vector Laboratories #BML-2202, Australia). Primary antibodies were omitted on control sections. Anti-type IIB primary antibody was detected by sequential incubation with biotin conjugated goat anti-mouse IgM (#B-9265, Sigma, Australia) (dilution 1:250) and streptavidin ALEXA488 (#S-11223, Molecular Probes, USA) (dilution 1:3000). Goat anti-rabbit ALEXA594 (#A-11012, Molecular Probes, USA) was used to detect rabbit anti-laminin primary antibody (dilution 1:250). Slides were mounted with PVA.

2.5. Morphometric Analysis

Non-overlapping images of the entire section of intact and denervated TA muscles stained with NADH-TR were captured using a Leica PM RBE microscope connected to a video camera (Hitachi HV-C20M) and a personal computer equipped with ImagePro Plus 4.0 (Microsoft) software. Immunohistochemically stained sections were examined under an Olympus IX50/IX70 inverted microscope and at least five non-overlapping digital images were captured using video camera Optronics MagnaFire—S99802 and MagnaFire 2.0 imaging software. Two images of the same region of the section were captured through the fluroscence microscope by using different filters for streptavidin ALEXA488 and goat anti-rabbit ALEXA594. The double images of the same microscope field stained with two different colors were merged into a single color image using MagnaFire 2.0 imaging software. On the merged image, type IIB myofibres were stained in green and all myofibres were sharply outlined by red fluorescent stained basal lamina. Cross sectional areas (CSA) of muscle fibres in denervated and intact TA muscles of wild-type and MLC/mIGF-1 mice were measured by tracing each individual myofibre using ImagePro Plus 4 imaging software. Care was taken to exclude any myofibre cut obliquely (i.e. with one axis being markedly longer than the other), since this would distort the CSA: such myofibres were present in low numbers in both transgenic and control mice. Denervated muscles of both strains were characterized by pronounced heterogeneity in the pattern of atrophy within

a single section: thus careful attention was paid to ensure that myofibre CSAs were measured in similar fields for both strains.

2.6. Statistical analysis

Values in the text and tables are reported as mean ± SD. Variables within the strains were compared using Student's *t*-test, while variables obtained by combined effect of strain (wild-type or transgenic) and treatment (intact or denervated) were compared using two-factor analysis of variance.

3. Results

3.1. Animal weights

There was no significant difference between the average weights of 12 weeks old intact wild-type (27.8 g ± 2.8 g) and MLC/mIGF-1 (31.0 g ± 2.8 g) mice. There was a slight reduction of the average weight at 28 days following denervation for both wild-type (25.3 g ± 1.7 g) and transgenic (29.5 g ± 0.6 g) mice, however, such weight reduction was not statistically significant for either strain as assessed by students *t*-test.

3.2. Histology of the TA muscle in wild-type and MLC/mIGF-1 mice at day 28 following denervation. Visualization of type IIB myofibres

Changes in the overall morphology of the TA muscles of wild-type and MLC/mIGF-1 mice following denervation were assessed on sections stained with NADH-TR. The blue colored reaction product obtained by reduction of tetrazolium (water-insoluble formazan pigment), binds to mitochondria and also to sarcoplasmic reticulum and the transverse tubular system and appears as granular deposits in the cytoplasm of the myofibres [22]. Staining with NADH-TR yields comparable results as staining with succinic dehydrogenase (SDH) [24], which is used to identify different fibre-types in skeletal muscle [25,26]. In all four experimental groups, type IIB myofibres were specifically identified using monoclonal antibodies against type IIB myosin [23]. In the present study we confirmed that in intact TA muscles of mice, myofibres with the weakest reaction for NADH-TR correspond to the fibres positive for IIB type myosin (Fig. 1 A, B). In denervated muscles, type IIB myofibres were no longer identifiable by NADH-TR staining as by day 28 after denervation myofibres have lost their shape (angularity), granularity of the cytoplasm and the normal reaction pattern for NADH-TR. This observation is in agreement with results obtained in 4 weeks denervated muscles stained with SDH [1,2]. Histological observation of the overall morphology of TA muscles of both wild-type and MLC/mIGF-1 mice at day 28 after denervation showed non-uniform atrophy of myofibres in different areas of the single muscle section, however overall the myofibre atrophy was more pronounced in wild-type mice (Fig. 1 C and D) compared to transgenic mice (Fig. 1 E and F). Detailed morphometric analysis of myofibre CSA was carried out to quantify the level of atrophy secondary to denervation in wild-type and MLC/mIGF-1 mice

3.3. Morphometric analysis of myofibres in TA muscle of intact and denervated wild-type and MLC/mIGF-1 mice

Mean CSAs of all types of myofibres and specifically type IIB myofibres were not significantly different between intact TA muscles of 12-weeks-old wild-type and MLC/mIGF-1 mice, however transgenic intact muscles contained a population of very large myofibres (not observed in wild-type muscles) and there was a slight shift of myofibre CSA distribution towards larger values in MLC/mIGF-1 compared to wild-type TAs (Table 1, Fig. 2 A–D and F). At day 28 following nerve transection, significant atrophy of myofibres was detected in both strains, however the extent of the decrease in myofibre mean CSA following denervation was less in transgenic compared to wild-type muscles (Table 1). Decline in the mean CSA of all types of myofibres and specifically type IIB myofibres after denervation was 59 and 61%, respectively, in wild-type mice and 37 and 40%, respectively, in MLC/mIGF-1 mice. The striking reduction in myofibre size is demonstrated by myofibre CSA distribution histograms, which show that small myofibres with CSA up to 2500 μm^2 comprise 39 and 36% of all myofibres in intact wild-type and transgenic muscles, respectively (Fig. 2 D) compared with 99 and 83%, respectively, in denervated wild-type and transgenic muscles (Fig. 2 E). A similar comparison for only type IIB myofibres showed that myofibres with CSA up to 2500 μm^2 comprise 13 and 11%, respectively, in intact wild-type and transgenic muscles (Fig. 2F) compared with 98 and 77% in denervated wild-type and transgenic muscles (Fig. 2G). Overall, the analysis of myofibre size distributions showed more larger myofibres persisting in denervated transgenic muscles compared to wild-type muscles (Fig. 2E and G).

4. Discussion

The present study demonstrates that muscle specific over-expression of the MLC/mIGF-1 transgene slightly decreases the rate of myofibre atrophy in short-term (28 days) denervated TA muscles. These data support our hypothesis that mIGF-1 is protective [20] and reduces atrophy of denervated myofibres.

In transgenic MLC/mIGF-1 mice [27] rat IGF-1 (exon 1-Ea isoform) is driven by the myosin light chain (MLC)-1/3 regulatory element that is expressed only in post-mitotic differentiated muscle cells (myotubes and myofibres) [28], with higher expression in fast-twitch (IIB, IIX and IIA)

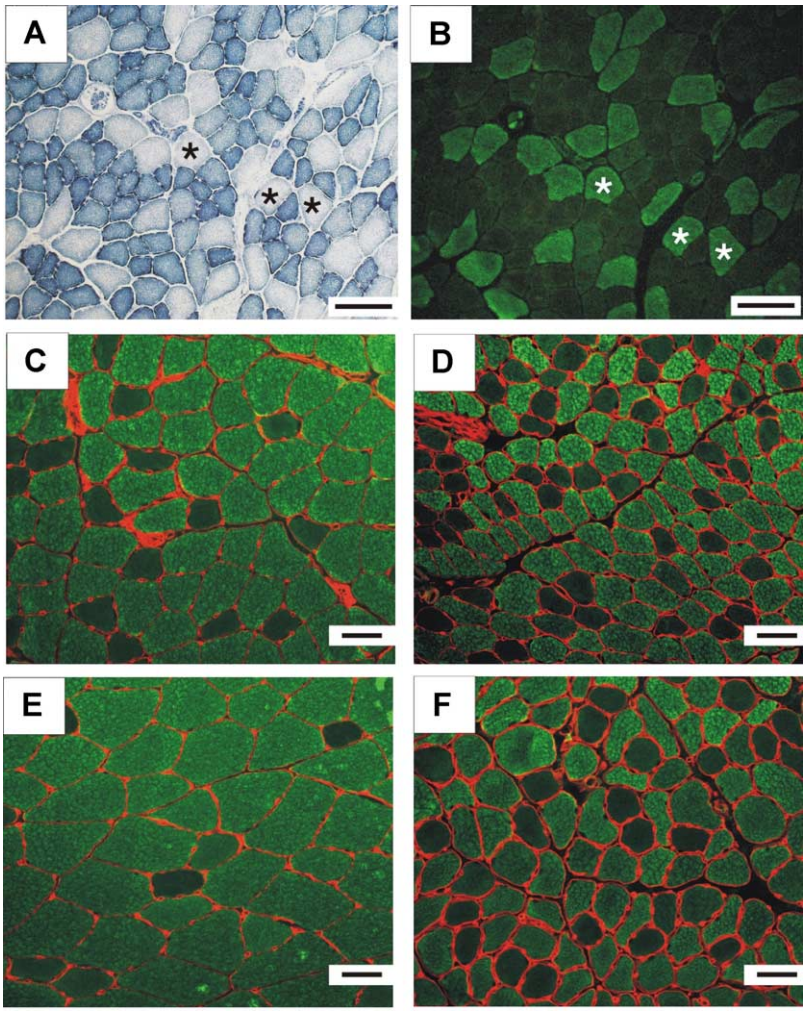

Fig. 1. Serial sections of the intact wild-type TA muscle stained with NADH-TR (A) and immunostained with antibodies against type IIB myosin (green fluorescence) (B). Myofibres with the weakest reaction for NADH-TR correspond to the myofibres positive for type IIB myosin (indicated with asterisk (*)). (A,B—bar indicates 100 μm). Intact (C,E) and denervated (D,F) TA muscles of wild-type (C,D) and MLC/mIGF-1 (E,F) mice immunostained with antibodies against type IIB myosin (green fluorescence) and PAN laminin (red fluorescence) which outlines myofibres. Atrophy of type IIB myofibres (green) and non-type IIB myofibres (not stained) is more pronounced in denervated wild-type TA (D) compared to MLC/mIGF-1 TA (F): this is shown quantitatively in Table 1. (C–F—bar indicates 50 μm).

myofibres then in slow-twitch (type I) myofibres [29]. Characterization of MLC/mIGF-1 mice showed hypertrophy of predominately fast-type muscles and such hypertrophy was maintained in aging (20 month old) animals, while muscle mass loss was detected in the wild-type mice [28]. The present study did not observe a significant difference in the average CSA of myofibres between intact TA muscles of wild-type and MLC/mIGF-1 mice at 3 months of age, although there was a shift of myofibre sizes towards bigger values in the transgenic mice.

4.1. mIGF-1 Slows down myofibre atrophy following denervation

It is well established that denervation causes progressive atrophy of myofibres primarily resulting from increased proteolysis and loss of contractile protein [30]. The rate of atrophy is especially high during the first month of denervation [31] with preferential atrophy of the fast type myofibres [5,32]. Based on the previous studies, which confirm hypertrophic action of mIGF-1 on myofibres and its ability to prevent atrophy of aging muscle, we hypothesized that over-expression of mIGF-1 will alleviate the process of atrophy in skeletal muscle following short-term (28 days) denervation.

In the present study, permanent denervation (transection of the sciatic nerve) caused severe atrophy of myofibres in TA muscles of both wild-type (∼60% loss in myofibre average CSA) and MLC/mIGF-1 (∼40% loss in myofibre average CSA) mice. The altered pattern of NADH-TR histochemical staining of denervated myofibres indicated progressive degenerative changes in the cell organelles, such as mitochondria and endoplasmic reticulum previously reported at the EM level [1,2,5]. The extent of denervation

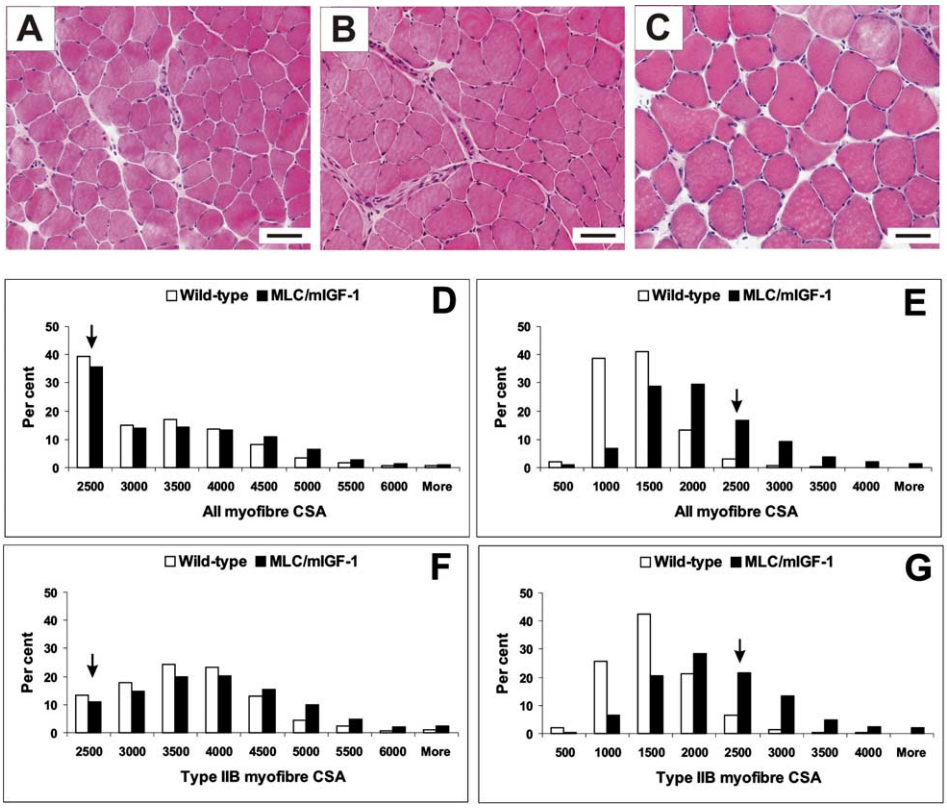

Fig. 2. Myofibre sizes in TA muscles of wild-type and MLC/mIGF-1 mice. Representative sections of intact TA muscles from wild-type (A) and MLC/mIGF-1 (B,C) mice stained with H&E showing myofibre size heterogeneity. Muscles of MLC/mIGF-1 mice contained a population of hypertrophic myofibres visible in only parts of the section (C), but not seen in other parts of the same section (B) and not observed in any wild-type muscles (A). (A–C—bar indicates 50 μm). Quantitation of myofiber size (CSA) distribution in intact (D,F) and denervated (E,G) TA muscles of wild-type and MLC/mIGF-1 mice. There is a slight shift of myofibre CSA towards larger values for all types of myofibres (D) and specifically type IIB myofibres (F) in intact TA muscles of MLC/mIGF-1 mice compared to wild-type mice. In denervated TAs a shift of all types of myofibre size (E) and specifically type IIB myofibre size (G) towards larger values is more pronounced in transgenic mice compared to wild-type controls, indicating the protective effect of mIGF-1 over-expression on myofibre atrophy following denervation: this protective effect of mIGF-1 was statistically significant for all types of myofibres, however not significant for type IIB myofibres (Table 1). Note the difference in scale in CSA between wild-type and MLC/mIGF-1 mice. The arrow indicates a reference point of CSA up to 2500 μm^2, since the denervated myofibres are much smaller.

induced muscle atrophy (assessed by analysis of CSAs of all myofibres) was significantly less (~30%) in MLC/mIGF-1 compared to wild-type TAs and the shift of myofibre CSA towards smaller values was less in transgenic MLC/mIGF-1 muscles. Since the highest level of MLC/mIGF-1 transgene expression was reported in type IIB myofibres [18], which are also most susceptible to denervation induced atrophy [5,7], we compared CSAs of specifically the type IIB myofibres in denervated TA muscles. Over-expression of mIGF-1 alleviated the atrophy of type IIB myofibres to approximately the same extent (~30%) as was seen cumulatively for all types of myofibres, however this difference in the decline of type IIB myofibre CSA between the wild-type and transgenic mice was not statistically significant. Since type IIB myofibres comprise about 40% of all myofibres in TA muscles of mice [26], the fact that the average rate of atrophy of all types of myofibres and specifically type IIB myofibres was ~30% less in transgenic mice compared to wild-type mice indicates that over-expression of the mIGF-1 transgene affects not only the type IIB myofibres in denervated MLC/mIGF-1 muscles.

While the severed sciatic nerve was clearly fully retracted, and no obvious nerve sprouting had occurred, it is critical to consider the issue of possible nerve re-growth from the severed proximal stump of the sciatic nerve (the distal end of the severed sciatic nerve degenerates). For re-innervation of TA to occur, any axons sprouting from the severed (retracted) distal stump of the sciatic nerve would have to grow to a considerable distance (within 4 weeks) and make functional synaptic connections with the myofibres in the TA muscle. This seems most unlikely in the present study. Lack of any re-innervation is supported by further marked atrophy of all myofibres at two months after denervation (data not shown) in transgenic and wild-type mice.

4.2. How does elevated IGF-1 protect denervated muscle from atrophy?

Denervated muscle is characterized by dramatically increased protein degradation, which results in the myofibre atrophy [30]. Since the protein content of myofibres depends

Table 1
Mean cross-sectional area (CSA) of all types of myofibres and type IIB myofibres in intact and denervated TA muscles of wild-type and MLC/mIGF-1 mice

	All myofibres		IIB Myofibres	
	Number measured	CSA (μm^2)	Number measured	CSA (μm^2)
Intact				
Wild-type ($n=8$)	4214	3009±370	1992	3454±343
MLC/mIGF-1 ($n=6$)	2864	3149±332	1324	3506±427
Denervated				
Wild-type ($n=10$)	5542	1229 ± 172	2832	1372±158
MLC/mIGF-1 ($n=7$)	4429	1990±333	2158	2106±323

(n) represents number of muscles analyzed. All numbers are based on the analysis of myofibres from randomly chosen fields of superficial and deep regions of TA muscles. Denervation significantly reduced mean CSA of all types of myofibres and specifically type IIB myofibres in both wild-type and MLC-mIGF-1 mice ($P<0.005$ calculated using Student's t-test). The extent of myofibre CSA reduction following denervation was compared between wild-type and MLC/mIGF-1 mice using two-factor analysis of variance. The extent of reduction of CSA of all types of myofibres was significantly ($P<0.05$) lower in MLC/mIGF-1 mice compared to wild-type mice. There was no significant difference between the extent of reduction of type IIB myofibre CSA between wild-type and transgenic mice following denervation.

on the balance between rates of protein degradation and protein synthesis, IGF-1 can have a protective effect on denervation induced muscle mass loss by decreasing proteolysis, increasing protein synthesis or both.

One of the mechanisms for mIGF-1 induced muscle hypertrophy is considered to be increased protein content of the myofibre via increasing myogenic cell proliferation and fusion [33]. A stimulatory effect of mIFG-1 on muscle regeneration has been proposed in MLC/mIGF-1 [34] and adult dystrophic mice [27]. However, studies of regenerating whole muscle autografts do not show superior regeneration (with respect to revascularisation, myoblast replication or onset and number of myotubes formed) in MLC/mIGF-1 grafts compared to wild-type grafts [21]. While these differences may be attributed to the different models of regeneration, they show that mIGF-1 may not enhance myogenesis in all situations. Another group showed earlier and increased expression of cyclin D, MyoD and myogenin with increased proliferation of putative myogenic cells in the gastrocnemius muscles of transgenic mice characterized by muscle specific over-expression of human IGF-1 driven by avian skeletal muscle α-actin promoter, compared to wild-type mice, within 3 days after sciatic nerve crush [35].

It is well established that denervation initially causes activation and sustained proliferation of satellite cells [4,6,36–40] followed by a steady decline in the number of satellite cells in the long-term denervated muscle [6]. Autoradiographic studies in mice show progressive loss of labeled nuclei adjacent to muscle fibres (presumed to be replicated satellite cells) from days 7 to 21 following denervation [37]. Several studies [5,36,38,41,42] suggest fusion of activated satellite cells into rare small myotubes, however the nuclear myofibre ratio does not change in denervated muscle (at least up to 21 days of denervation), indicating that activated satellite cells fail to fuse to existing myofibres [37]. Overall, a contribution of satellite cells to sustaining the mass of denervated muscle is doubtful and the crucial factor that determines the restorative capacity of the muscle is innervation (discussed in [5]). Our observations on 28 day denervated muscle sections of wild-type and MLC/mIGF-1 mice immunostained with antibodies against desmin show no evidence of small myotubes and do not support fusion of activated satellite cells into myotubes (Shavlakadze et al. unpublished observations). Although small myotube-like myofibres with centralized nuclei were occasionally seen in the denervated muscle tissue, they did not exhibit intensive desmin staining characteristic for the young myotubes [43], which suggests that they are more likely to be atrophic myofibres, which acquire central nucleation due to decreased CSA. On the basis of these observations it seems unlikely that over-expression of IGF-1 in transgenic mice would alleviate atrophy of skeletal muscles through fusion of activated satellite cells.

Muscle hypertrophy can occur in the absence of satellite cell proliferation, due to increased protein synthesis within myofibres [44]. Activation of protein synthesis results from activation of PI3-kinase/Akt/mTOR pathway, which represents a signalling pathway downstream of the IGF-1 receptor [14,45]. In mice, transfection of TA muscles with a constitutively active form of Akt (Akt is a downstream target for the activated IGF-1 receptor) had a protective effect on myofibre atrophy following 7 day denervation and it was suggested that this protection is due to increased protein synthesis [45]. A direct effect of mIGF-1 transgene over-expression on myofibres was also shown in mice after gamma irradiation (this inhibits proliferation of satellite cells and thus causes muscle mass loss over time) where muscles transfected with the mIGF-1 transgene lost ~50% less mass compared to control non-transfected muscles at 4 month after irradiation [46]. Our recent observations that mIGF-1 over-expression protects myofibres from necrosis in young (up to 1 month) mdx mice during the acute onset of dystrophic muscle degeneration [20] may also reflect altered protein metabolism.

Nerve transection causes increased muscle net protein breakdown and loss of contractile elements [30] and it is suggested that such increased proteolysis is due mainly to acceleration of the ubiquitin-proteosome dependent pathway [30,47–49]. Inhibitory effects of IGF-1 on the ubiquitin-proteosome protein degradation pathway in skeletal muscles have been reported in different experimental models characterized by increased proteolysis (e.g. burn injury [50], sepsis [51] and dexamethasone treatment [52]). Furthermore, it has recently been demonstrated that

over-expression of the mIGF-1 transgene prevents skeletal muscle atrophy in mice with cardiac dysfunction by blocking ubiquitin-proteasome targeting of specific structural proteins (N. Rosenthal, personal communication). IGF-1 induced suppression of the atrophy related genes MAFbx and MuRF1, which encode ubiquitin ligases has been also shown in dexamethasone treated C2C12 myotubes and short-term denervated muscles [53].

In summary, muscle specific over-expression of mIGF-1 slightly alleviates atrophic changes in short-term (28 days) denervated skeletal muscle, and it seems that this protective effect is due to a direct action on protein metabolism in mature myofibres.

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