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Phenotypic Expression of IGF Binding Protein Transcripts in Muscle, *in Vitro* and *in Vivo*

S. Bayol,* P. T. Loughna,†,[1] and C. Brownson*,[2]

**School of Health and Sports Science, University of North London, Holloway Road, London N7 8DB, United Kingdom; and †Molecular Physiology Unit, Department of Veterinary Basic Sciences, Royal Veterinary College, London, NW1 0TU, United Kingdom*

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The actions of the insulin-like growth factors (IGF-I and IGF-II) which are essential components of skeletal muscle growth are mediated via their receptors and modulated by six binding proteins (IGFBPs). We studied IGFBP transcripts in C2C12 cell cultures and in adult control and denervated gastrocnemius muscle. IGFBP-2, -4, -5, and -6 were detected in C2C12 cells. IGFBP-6 mRNA levels remained unchanged, IGFBP-2 levels decreased and IGFBP-4 and -5 increased over 1, 5, and 9 days after serum reduction. In a range of adult muscles studied, IGFBP-4 mRNA levels were similar, IGFBP-5 was present at different levels in slow and fast muscles and IGFBP-6 had the lowest expression in the tibialis anterior. Denervation resulted in dramatic up-regulation of IGFBP-4 and -5 transcripts but there was no change in IGFBP-6. These results suggest that either lack of neural input and/or mechanical loading, both of which contribute to muscle atrophy, affect IGFBP expression.

Key Words: **IGFBP; C2C12 cells; skeletal muscle; denervation; IGF.**

The insulin like growth factors (IGF-I and IGF-II), which are members of the family of insulin-related peptides, have essential roles in growth and development (1, 2). The endocrine actions of the growth hormone/IGF axis are well documented but the IGFs, which are synthesised and secreted by many cell types, also regulate cell growth via autocrine/paracrine mechanisms (for a review see 3). It is evident from many studies that the mechanisms by which the IGFs act are complex and involve not only the two IGFs themselves but also two cell membrane receptors and a family of at least six binding proteins (IGFBP-1-6) which are responsible for modulating the actions of the IGFs (3, 4). Until recently most of the evidence for IGFBP function has been obtained from *in vitro* studies using different cell lines and culture conditions and the current picture of their role is unclear. Evidence from *in vitro* studies suggests that IGFBP-2, -4, and -6 largely inhibit IGF action, IGFBP-5 enhances its action and IGFBP-1 and -3 can be either inhibitory or stimulatory (5). IGFBP-5, the major binding protein in skeletal muscle (6), associates with the extracellular matrix which in fibroblasts and smooth muscle cells (7) reduces its affinity for the IGFs thus potentiating the growth response.

The IGFBPs are widely expressed in adult tissues but there is considerable variation in the type and extent of binding protein expressed. They each have different roles and their function may be cell/tissue dependent. At least some of the IGFBPs have IGF-independent functions. Their activity is regulated by IGFBP-specific proteases, phosphorylation and in some cases (IGFBP-2, -3, and -5) by association with the extracellular matrix (for reviews see 3, 5). Currently the molecular mechanisms by which the IGFBPs inhibit or enhance the signalling roles of the IGFs is unclear.

Myogenesis and skeletal muscle growth and regeneration are dependent on the IGFs (8, 9) which also have a role in moderating the age related loss of skeletal muscle function (10). *In vitro* studies have suggested that specific IGFBP genes are expressed in a developmentally specific order during early myogenesis but this varies in different muscle cell types (11, 12). The differential expression of the IGFBPs in muscle cell cultures suggests that they play individual roles in early muscle development. Following formation of myofibres skeletal muscles further differentiate into adult fast and slow fibre types either just prior to or shortly after birth. There is considerable evidence for a role of IGF-I in post-natal muscle fiber hypertrophy. Recent *in vitro* studies of C2C12 cells stably trans-

[1] To whom correspondence should be addressed. Fax: 0171 3881027. E-mail: ploughna@rvc.ac.uk.

[2] Present address: Division of Biomolecular Sciences, GKT School of Biomedical Sciences, King's College London, Guy's Campus, London Bridge, London SE1 1Ul, United Kingdom.

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fected with IGF-I and *in vivo* studies of rat latissimus dorsi muscle injected with a plasmid encoding IGF-I demonstrated that IGF not only influenced muscle hypertrophy but also phenotype (14). IGF levels in adult fast and slow muscles are similar but currently there is little documentation of IGFBP levels in different adult skeletal muscle types.

Muscle fiber mass and phenotype is not fixed postnatally but can be changed by various external stimuli which lead to alterations in contractile, transport and metabolic proteins (13). Muscle hypertrophy caused by passive stretch (11, 13, 15), endurance training (16) and muscle regeneration (17) but not disuse atrophy in rats was accompanied by an increase in IGF-1 mRNA. Denervation (18) resulted in an increase in IGF-I mRNA levels in fast muscles (plantaris and gastrocnemius) and decreased levels in slow (soleus) and induction of IGF-II mRNA in all three muscles within 2–3 days. However, the effect of external stimuli such as changes in activity or hormones, mechanical stretch or disuse on the expression of the various IGFBPs in skeletal muscles and the consequences for IGF-I activity is currently unknown.

In the present study we investigated the expression of the various IGFBPs in late muscle in C2C12 cell cultures and adult skeletal muscles of different fiber type and changes in this expression following denervation of adult muscle.

METHODS

Animals and experimental protocol. Denervation of male Wistar rats was carried out by sectioning the sciatic nerve at the level of the upper thigh region in one hind-limb of growing rats (200–250 g). Surgical procedures were carried out aseptically under halothane/oxygen mixture anaesthesia. After two days the animals were sacrificed and gastrocnemius muscles removed from experimental and contra-lateral control limbs and rapidly frozen in liquid N_2.

Cell culture. Cells from a C2C12 mouse skeletal muscle cell line were plated out in 35 mm diameter cell culture dishes at a density of 1.5×10^4 cells/cm^2. They were incubated at 37°C in proliferation medium (DMEM + Glutamax; 10% foetal bovine serum; 1% penicillin/streptomycin/fungizone [all Life Technologies]) and the medium was changed every 2 days until confluence was reached. The cells were then incubated in differentiation medium (DMEM + Glutamax; 2% foetal bovine serum; 1% penicillin/streptomycin/fungizone) which was changed every 2 days. The cells were harvested by removing the media, washing the cells in Dulbecco's PBS (Life Technologies) and then adding 1 ml RNAzol (Biogenesis) at three different time points: 1, 5, and 9 days after the differentiation medium was first added. Myotubes were first distinguishable after 5 days and a well established network was present after 9 days.

RNA isolation and Northern blotting. Total RNA was extracted from cell culture samples and homogenised muscle using RNAZol-B (Biotec X) according to the manufacturer's instructions. RNA (10 μg) denatured with glyoxal/DMSO at 50°C was separated by electrophoresis in 1% agarose gels in 10 mM sodium phosphate buffer, pH 7.0. RNA was transferred to nylon membranes (MSI, Osmonics, Inc.) and heated for 2 hours at 80°C. Prehybridisation and hybridisation procedures were carried out at 48°C in 50% formamide. cDNA fragments for IGF-I and II (ATCC) and IGFBPs 1-6 (Kindly supplied by Dr. S. Shimasaki), used to analyse the muscle tissue, were isolated from plasmids after digestion with the appropriate restriction enzymes. A 18s rRNA probe was used as an internal control. Fragments were radioactively labelled using the random priming method with α-[^{32}P]-dCTP and a multiprime labelling kit (Amersham Int. plc.). Filters were given a final wash in 0.2 × SSC, 0.1% SDS for 5 minutes at 60°C. Blots were exposed to X-Omat XAR-5-F6613 film.

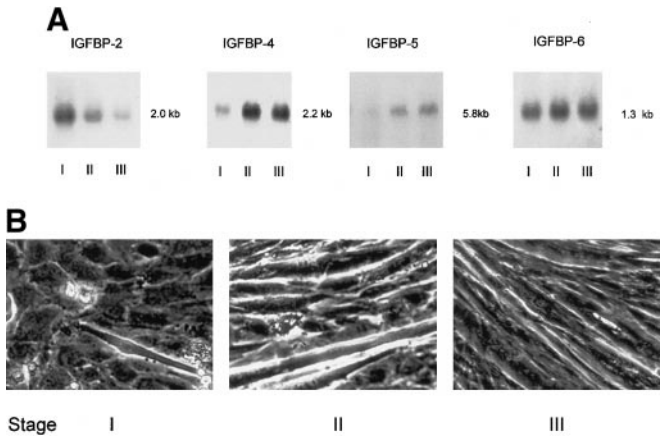

FIG. 1. Northern analyses of IGFBP transcripts in C2C12 myoblast cultures at three different stages of differentiation. Northern blots of IGFBP-2, -4, -5, and -6 mRNAs and cell cultures at 1 (I), 5 (II) and 9 (III) days after serum reduction are shown.

Data analysis. The intensity of the signal on the developed X-ray was assessed by image analysis. Results were expressed as a ratio of the rRNA present and given as the mean ± SD. The data for the comparison of different muscles was analysed by Anova and Tukeys method and the denervation data was analysed by two-tailed Student's *t*-test and considered significant if $P < 0.05$.

RESULTS

Expression of IGFBP cDNAs in Cell Culture

Previous work carried out on myoblasts and myotubes immediately postfusion had indicated that the expression of the IGFBPs was different in different cell culture lines (8). We determined the distribution of the IGFBP mRNAs in cultures of C2C12 muscle cells at 1 day (stage I), 5 days (stage II), and 9 days (stage III) after serum reduction. From Fig. 1B it can be seen that at 1 day after serum reduction myoblasts were still prominent, whereas after 5 and 9 days 50 and 90% fusion of the myoblasts, respectively, had occurred. Northern analyses show that IGFBP-2, -4, -5, and -6 mRNAs were present at various stages of culture whereas no IGFBP-1 or -3 transcripts were detected (Fig. 1A). IGFBP-2 transcript levels decreased, whereas IGFBP-4 and -5 increased at each stage of development. Levels of IGFBP-6 mRNA remained unchanged at each time-point studied.

Expression of IGFBP cDNAs in Adult Skeletal Muscle Tissues

Various rat adult skeletal muscles of different fiber type composition were analysed for IGFBP mRNA lev-

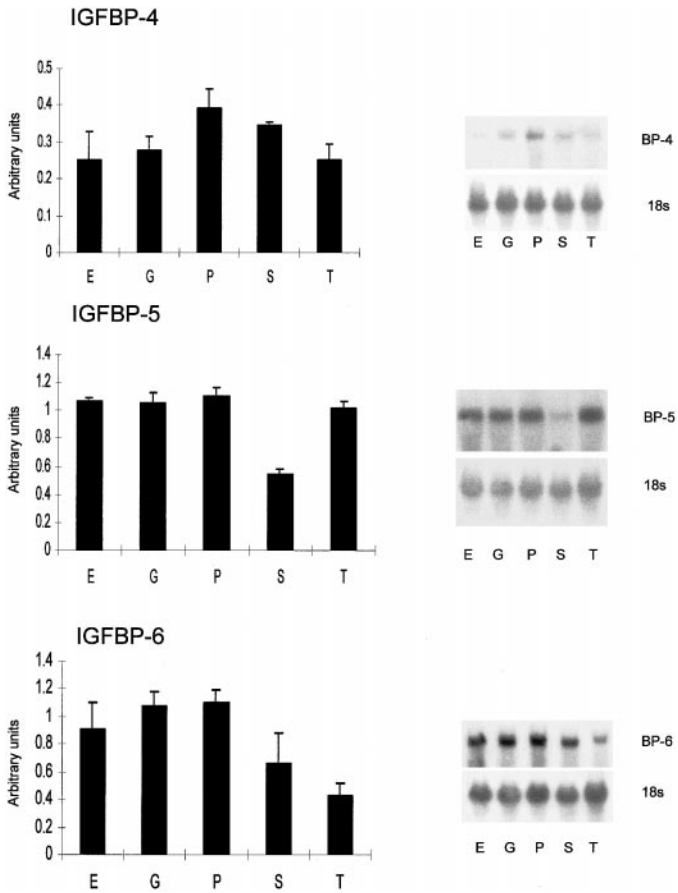

FIG. 2. Northern analyses of IGFBP transcripts in various muscles of the rat. Northern analyses of IGFBP-4, -5, and -6 mRNAs and control 18s rRNA bands are shown together with histograms of the image analysis data from the mRNAs as a ratio of the corresponding rRNA ($n = 3$). E, extensor digitorum longus; G, gastrocnemius; P, plantaris; S, soleus; T, tibialis anterior.

els. IGFBP-1 transcripts were not detected in any of the adult skeletal muscles studied and IGFBP-2 and -3 mRNAs were only present at low levels and were not significantly different between the different muscles ($P = 0.319$ for IGFBP-2 and $P = 0.777$ for IGFBP-3). All three transcripts were present at high levels in liver extracts as was IGFBP-4 mRNA (results not shown). Figure 2 shows the Northern blots and histograms of the data obtained from image analysis of the IGFBP mRNAs. It can be seen that transcripts of IGFBP-4, -5, and -6 were readily detectable in all adult skeletal muscles analysed although the level of expression varied between muscles. IGFBP-4 transcript levels were not significantly different between the muscles studied ($P = 0.214$). IGFBP-5 mRNA was found be expressed at significantly different levels in the muscles examined ($P = 0.0036$) and Tukeys method showed that the levels in the slow soleus were significantly lower than the other fast phasic muscles. On the other hand, although the IGFBP-6 transcript showed significantly different levels between the muscles examined ($P = 0.046$) no individual muscle was found to differ significantly from the others.

IGFBPs in Denervated Muscle

Short-term denervation as used in this study avoids the complications of fiber degeneration and subsequent regeneration associated with longer term denervation while still allowing rapid changes in gene expression to be documented. The denervated muscle used in this study was the mixed gastrocnemius muscle and the contralateral muscles were used as controls. IGFBP-4, -5, and -6 mRNAs were readily detectable in these muscles. The Northern blots (Fig. 3A) and histograms (Fig. 3B) of the image analysis data of the IGFBP transcripts following denervation of this muscle for 2 days show that there is little change in the levels of IGFBP-2, -3, or -6 mRNAs. However there is a dramatic up-regulation of IGFBP-4 ($P < 0.02$) and IGFBP-5 ($P < 0.05$) mRNAs in denervated muscles to 319% and 302% of control contralateral muscles respectively.

DISCUSSION

The IGFs have an important role in muscle development and growth (12). As potential regulators of IGFs the IGFBPs would also be expected to be involved in

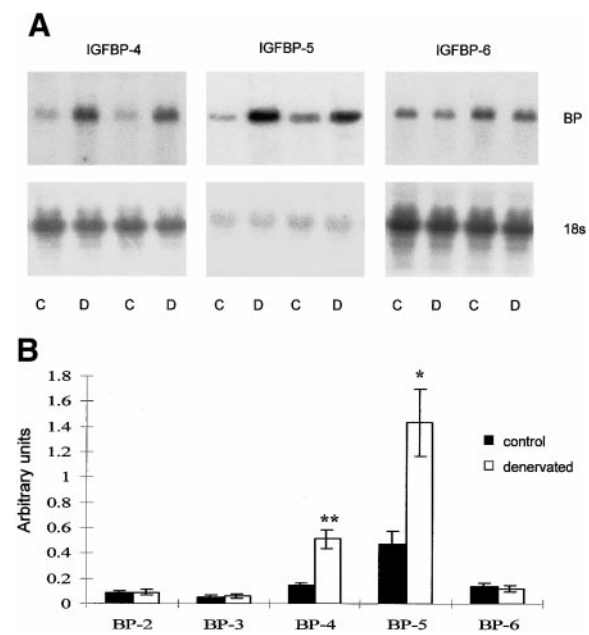

FIG. 3. Northern analyses of IGFBP transcripts in control and denervated gastrocnemius muscle. Northern analyses of IGFBP-4, -5, and -6 mRNAs and control 18s rRNA bands (A) are shown together with histograms of the image analysis data from IGFBP-2, -3, -4, -5, and -6 mRNAs as a ratio of the corresponding rRNA (B) ($n = 5$). C, contralateral control muscles; D, denervated muscles.

muscle development, maintenance and regeneration. Previous reports have shown that the IGFBPs are expressed to different extents in different muscle cell lines and changes in their expression correlate with those of the IGFs during myoblast proliferation and fusion (12). For example, IGFBP-4, -5, and -6 are expressed by rat L6 and the related L6E9 cell lines (19). In contrast, mouse C2 cells predominantly express IGFBP-5 (20). We have shown that C2C12 mouse cell lines, which are derived from C2 cells, express IGFBP-2, -4, -5, and -6 at fusion and in differentiated cells at time points which are later than those previously studied. This result is in line with a previous investigation where IGFBP-2 and two additional uncharacterised IGFBP transcripts were detected in C2C12 cells (21). In our study of C2C12 cells the expression of the various binding proteins appeared to be developmentally regulated. This is in line with the study of Rousse *et al.* (22) in the same cell line in which IGFBP-2 was prevalent in proliferating myoblasts and IGFBP-5 was stimulated during differentiation and also with other studies using different cell lines.

Skeletal muscles can have distinctly different phenotypes with regard to mechanical and metabolic properties which are dependent upon their functional role. The expression of IGFBPs in different adult muscles has not been extensively investigated. Of the muscles used in the present study, EDL, plantaris and TA are fast phasic and composed mainly of type 2 fibers. Gastrocnemius is a mixed muscle containing a large proportion of type 2A (fast oxidative glycolytic) and type 2B (fast glycolytic) and a smaller proportion of type 1 (slow oxidative) fibers and soleus is a slow postural muscle composed almost entirely of type 1 and type 2A fibers. The levels of IGF-I expression are not significantly different between muscles (16) or the satellite-derived myoblasts of muscles (23) of distinctly different phenotype such as the slow postural soleus and the fast phasic plantaris. This suggests that concentrations of locally produced IGF-I which may act in an autocrine/paracrine manner does not differ between the two muscle types. Furthermore, all skeletal muscles will be exposed to a similar IGF-I endocrine environment. Thus differential effects of IGF-I in muscles of different phenotype may well be attributable to varying levels of the IGF-I receptor and/or the IGFBPs. Therefore, it is of interest that our results indicate that IGFBP-5 is present at lower levels in slow soleus muscle than the fast phasic muscles potentially mediating a different response to the IGFs. However, such specificity is not evident for the IGFBP-4 and -6 transcripts. The expression of these latter factors may depend on the individual muscle and possibly its pattern of activity.

Denervation of skeletal muscle results in rapid atrophy and changes in contractile protein gene expression and subsequently leads to muscle fiber degeneration. Little is known about the mechanisms responsible for the changes in gene expression which occur following denervation but levels of IGF transcripts are known to be altered (18). Further, administration of IGF improves muscle fiber morphology and aids muscle recovery by accelerating reinnervation following nerve section (24). We have demonstrated that transcripts of IGFBP-4 and -5 are up-regulated by approximately threefold in gastrocnemius muscle following denervation. In all systems investigated so far IGFBP-4 has an inhibitory effect on the IGFs. On the other hand, IGFBP-5 has been shown to be a major binding protein associated with the extracellular matrix of skeletal muscle which appears to enhance the binding of IGF-I with its receptor (11). These results could suggest that IGFBP-5 is up-regulated in response to either a lack of neural input or decreased mechanical loading which together contribute to muscle atrophy. The latter proposal is supported by the study of Awede *et al.* (25) in which a similar increase in IGFBP-5 transcript levels was observed in a rat model of hind limb unloading (atrophy) whereas as a decrease was observed with overload hypertrophy. IGFBP-5 could be involved in the changes in fiber size that occur in these models of atrophy and hypertrophy. Overload hypertrophy is associated with changes to a slower phenotype (26) whereas many studies have shown that there is an up-regulation of fast muscle type genes with both disuse and denervation atrophy (27, 28). Thus, IGFBP-5 could play a role in the regulation of qualitative changes in phenotype. The decrease in IGFBP-5 levels with overload would then correspond with the lower levels of this binding protein which we observed in soleus muscle and the up-regulation observed with denervation with the higher levels observed in fast muscles. The reasons for the up-regulation of the both IGFBP-4 and -5 transcripts which appear to exert opposite effects on IGF-I activity are presently unclear but again emphasises the complexity of this system.

The mechanisms associated with the initiation of the IGF signalling pathway, which is universally involved in growth and development and involves the different IGFs, two cell surface receptors and six binding proteins, have not been clearly elucidated. Skeletal muscle provides an ideal system in which to study such mechanisms due to the dramatic morphological changes which occur during myogenesis and the plasticity of adult skeletal muscle.

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