Characterisation of the IGF system in a primary adult human skeletal muscle cell model, and comparison of the effects of insulin and IGF-I on protein metabolism

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Abstract

In an attempt to address the complex and clinically challenging question of the causes of muscle wasting in patients with cachexia, we have developed a primary adult human skeletal muscle cell model. The cultured cells were characterised by immunocytochemistry using antibodies to the myofibrillar protein constituents desmin and titin. Myotube formation was confirmed biochemically by a fourfold increase in the activity of the muscle-specific enzyme creatinine kinase, and myoblast withdrawal from the cell cycle, which is essential for terminal differentiation, was associated with progressive retinoblastoma protein dephosphorylation. Having successfully confirmed the phenotype of these adult human muscle cells, we assessed their interaction with the insulin-like growth factor (IGF) system. IGF-I is known to stimulate myoblast survival, proliferation and differentiation in cell lines, and, like insulin, is a potent anabolic agent in the regulation of protein metabolism. We have shown that IGF-I stimulated both replication and differentiation of myoblasts, whilst fibroblast growth factor-2 stimulated replication but inhibited differentiation. Examining the IGF system during the

process of terminal differentiation, we found that both myoblasts and myotubes expressed insulin, IGF-I and insulin-IGF-I hybrid receptors, with the levels of all three receptor types increasing on differentiation. The cells also produced a wide range of IGF binding proteins (IGFBPs) including IGFBP-2, IGFBP-4 and abundant IGFBP-3, which has not been shown to be produced by any other skeletal muscle cell line examined to date. Both insulin and IGF-I had anabolic effects on myotube protein metabolism at physiological concentrations. Insulin was more potent than IGF-I: use of the IGF analogue long R³ IGF-I demonstrated that the effects of exogenous IGF-I on protein metabolism were not affected by the high levels of endogenous IGFBP production. In summary, we have developed and characterised a clinically relevant *in vitro* model with which to address the aetiology of muscle wasting associated with chronic catabolic conditions, and we anticipate that future work will enable the development of novel, effective therapeutic interventions.

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Introduction

Cachexia is associated with many chronic severe illnesses and is characterised by anorexia, weight loss, and an altered metabolism with increased energy expenditure (Calman 1982). There is an impaired ability to conserve lean body mass which results in muscle wasting (Wilmore 1991), affecting respiratory muscles, mobility and performance status. Even small amounts of weight loss (less than 5%) significantly worsen the prognosis of patients with cancer (DeWys *et al.* 1980). Cachexia has been described as 'among the most visible and devastating consequences of several human diseases' (Nabel & Grunfeld 1996) yet at present no effective treatment is available.

We and others have demonstrated that the insulin-like growth factors (IGFs) are unique in their ability to stimulate myoblast survival and proliferation, as well as differentiation (Florini *et al.* 1995, Stewart & Rotwein 1996). Supporting these *in vitro* data, the relevance of the IGF system has been demonstrated *in vivo*, where IGF-I, IGF-II and IGF-I receptor knockout studies result in neonatal lethality primarily as a consequence of skeletal muscle hypoplasia (Liu *et al.* 1993, Stewart & Rotwein 1996). Although there is much *in vitro* evidence to support the anabolic roles of insulin and IGF-I in regulating skeletal muscle protein metabolism, many of these studies have used immortalised skeletal muscle cell lines originating from the rat (L6) or mouse (C2) (Roeder *et al.* 1988),

or, where primary cultures have been used, they have been derived from fetal tissue (Hill *et al.* 1986, Frost *et al.* 1997, Harper *et al.* 1987). Additionally, many of the above experiments have been performed on myoblasts rather than the more physiologically relevant myotubes (Hill *et al.* 1986, Frost *et al.* 1997). While the role of the IGF binding proteins (IGFBPs) in modulating the metabolic responses of skeletal muscle to IGF-I has not been addressed, their release and relevance has been demonstrated in murine C2 myoblasts, where IGFBP-5 reduced differentiation whilst maintaining myoblast cell survival (James *et al.* 1996).

IGF-I and insulin have been used in attempts to treat cachexia in human disease (Wolf *et al.* 1992, Lieberman *et al.* 1994, Lee *et al.* 1996) and in animal models (Douglas *et al.* 1991, Ng *et al.* 1992, Tomas *et al.* 1994), but the results of such studies have been variable, re-emphasising the need for an improved understanding of the pathophysiology of muscle wasting in order to develop better therapeutic interventions. In order to achieve this end, an *in vitro* model which is relevant to the study of patients with cachexia is required. We therefore developed a primary adult human skeletal muscle cell culture system, and characterised the cells with regard to their morphological and biochemical parameters of differentiation. Since the IGFs appear to be critical for the formation of multinucleate myotubes, we examined the IGF system during the process of terminal differentiation. Results reported here demonstrate that the cells express insulin, IGF-I and insulin-IGF-I hybrid receptors and produce a wide range of IGFBPs, surprisingly including abundant IGFBP-3. Having characterised the system, we confirmed the functionality of the cells by demonstrating anabolic effects of insulin and IGF-I on protein metabolism, with insulin being more potent at physiological concentrations. Thus we have developed and characterised a sophisticated *in vitro* model with which to address the complex problem of muscle wasting associated with chronic catabolic conditions.

Materials and Methods

Subjects

Perioperative skeletal muscle biopsies were taken from the anterior abdominal wall of women between 18 and 60 years of age undergoing elective gynaecological operations for non-malignant conditions. Those with known metabolic or endocrine disease were excluded. Cells derived from 30 women with a mean age of 43.3 years (standard deviation 8.0 years) were used in these studies. A smaller number of biopsies were also taken from the anterior abdominal wall of subjects undergoing upper gastrointestinal operations. The study was approved by the local Ethical Committee, and all subjects gave written, informed consent.

Media

All chemicals and media are from Sigma (Poole, Dorset, UK) unless otherwise stated. The skeletal muscle growth medium (GM) is a modification of that of Ham *et al.* (1988): MCDB-131 with L-glutamine, 50 IU/ml penicillin, 5 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 0.4 µg/ml dexamethasone, 0.5 mg/ml bovine serum albumin (BSA), 0.5 mg/ml fetuin, and 10% fetal calf serum (FCS) (GIBCO, Paisley, UK). Initially, 50 µg/ml insulin (Novo Nordisk, Crawley, West Sussex, UK) was included in the GM, as suggested by Ham *et al.* (1988); we subsequently found that cells grew equally well in the absence of insulin. Skeletal muscle fusion medium (FM) consisted of minimum essential medium (MEM)-alpha modification with 50 IU/ml penicillin, 5 µg/ml streptomycin, 1% L-glutamine and 2% FCS (GIBCO). Fibroblast growth medium was Dulbecco's modified Eagle's medium (DMEM) with Glutamax, with 50 IU/ml penicillin, 5 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 10% FCS. For serum-free medium (SFM), the FCS was omitted from the skeletal muscle FM/fibroblast GM, and 0.1% BSA was added.

Isolation of primary adult human myoblasts

Primary skeletal muscle cell cultures were derived according to a modified method of Blau and Webster (1981). Briefly, biopsies were dissected in phosphate-buffered saline (PBS) to remove all visible fat and connective tissue, and were then chopped finely (1 mm³ pieces), prior to being digested in trypsin/EDTA (T/E solution at final concentrations of 0.05% trypsin and 0.02% EDTA in PBS) for 15 min. The supernatant was removed and mixed with FCS (GIBCO), centrifuged at 1300 r.p.m. for 5 min and the pellet resuspended in skeletal muscle GM. This process was repeated an additional three times. The cell suspensions from each T/E digestion were pooled and plated in Nunclon flasks pre-coated with 0.2% gelatin type B. Cells were allowed to settle for up to 72 h before the first medium change, and were passaged upon attaining 80–90% confluency. Cells at passages 3–5 were used for all experiments. When confluent, experimental cells were washed twice with PBS prior to addition of FM. All experiments were carried out following 7 days in FM, unless otherwise stated.

Human fetal myoblasts

Human fetal myoblasts derived from a 22-week-old fetus were purchased from Clonetics (Wokingham, Berks, UK).

Primary human fibroblasts

Primary adult human skin fibroblasts (which were a kind gift from Dr Jane Samson, Bristol Royal Infirmary) were derived from epidermal biopsies.

Characterisation of primary human myoblasts

Immunocytochemical characterisation was carried out on cells grown on glass coverslips and on cytopins of cell suspensions. Mouse monoclonal antibodies to the myofibrillar protein constituents desmin (Dako, Ely, Cambs, UK) and titin (Novocastra, Euro-Path Ltd, Bude, Cornwall, UK) were used at dilutions of 1:100, followed by specific secondary antibodies. Nuclei were counterstained with haematoxylin for clarity, in order that the ratio of positively staining myoblasts to negatively staining fibroblasts could be easily quantitated. In addition to the cellular morphology and immunocytochemistry, differentiation was also characterised biochemically by measuring the activity of the muscle-specific enzyme creatinine kinase (CK) in Tris–MES–Triton cell lysates (50 mM Tris–MES pH 7.8 with 1% Triton-X 100), using the 'CK-10' kit (Sigma). Samples were stored at -80°C and assayed within 14 days of collection. Enzymatic activity was normalised to total protein content, as determined by the bicinchonic acid (BCA) protein assay according to the manufacturer's instructions (Pierce, Chester, UK).

Cell cycle arrest was determined by assessing the retinoblastoma protein (Rb) status of myoblasts and myotubes. Cells were lysed (10 mM Tris–HCl pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 100 μM Na orthovanadate, 1% Triton-X 100, with 10 μl phenylmethylsulphonylfluoride (PMSF) solution/ml (1 mg PMSF in 0.57 ml ethanol) added immediately before use), and the lysates were subjected to 6% SDS polyacrylamide gel electrophoresis (PAGE) under denaturing, reducing conditions. Following electro-transfer to nitrocellulose ('Hybond-C' membrane, Amersham Life Science, Little Chalfont, Bucks, UK), non-specific binding sites were blocked in 5% non-fat dried milk for at least one hour at room temperature. Blots were then incubated with a 1:500 dilution of phospho-specific goat anti-Rb antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with horseradish peroxidase conjugated anti-goat immunoglobulin, proteins were detected by enhanced chemiluminescence (ECL detection reagents, Amersham Life Science) followed by autoradiography. All experiments described were performed following 7 days in FM with or without IGF-I or fibroblast growth factor-2 (FGF-2) as positive and negative differentiation controls respectively (Florini *et al.* 1995).

IGF-I and insulin receptor status

To assess the IGF-I and insulin receptor status, cells were lysed (as detailed above) and the protein concentration determined by BCA assay according to the manufacturer's instructions. Samples were boiled for 5 min in Laemmli reducing sample buffer (Sigma) and loaded according to their protein content on an 8% polyacrylamide gel.

Figure 1 Phase contrast light microscopy of primary human myoblasts and myotubes showing progressive differentiation in fusion medium (magnification $\times 20$).

Following electrophoresis, the proteins were electro-transferred onto a nitrocellulose membrane. Immunoblotting was carried out using primary antibodies to the insulin or the IGF-I receptor β -subunits (Santa Cruz Biotechnology) at dilutions of 1:500. Following incubation with secondary antibodies, proteins were detected by ECL followed by autoradiography (X-Omat LS, Kodak, Sigma).

For analysis of the insulin–IGF-I hybrid receptor status, 200 μg total cell lysates isolated as above were immunoprecipitated using the anti-IGF-I receptor antibody conjugated to protein A agarose beads (Calbiochem, Nottingham, UK). After mixing for 3 h at 4°C , samples were boiled in Laemmli buffer and subjected to SDS PAGE followed by electro-transfer. The nitrocellulose membrane was then immunoblotted using the anti-insulin receptor antibody as above.

Figure 2 Immunocytochemical characterisation of myotube cultures and negative staining of fibroblast cultures using (A) anti-desmin and (B) anti-titin antibodies (magnification $\times 20$).

IGFBP profiles

IGF binding protein (IGFBP) profiles in conditioned media were examined by SDS PAGE followed by Western ligand and immunoblotting. Samples were denatured by boiling for 5 min in non-reducing sample buffer, then separated by 12.5% SDS PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked successively in solutions containing 0.8% Tris-HCl pH 7.4 with 0.05% Nonidet P40 (30 min), 3% bovine serum albumin (90 min) and 0.8% NaCl and 0.2% Tween 20 (20 min), then probed using ^{125}I -IGF-I and ^{125}I -IGF-II, and, after washing, was exposed to autoradiographic film (Coulson *et al.* 1991). Immunoblotting was subsequently carried out using an in-house anti-IGFBP-3 rabbit polyclonal antibody (1:15 000; Cwyfan Hughes *et al.* 1995), or an IGFBP-2 antibody (1:2000, TCS Biologicals Ltd, Buckingham, UK), or an IGFBP-4 antibody (1:1000, TCS Biologicals Ltd), followed by ECL.

Radioimmunoassays for IGF-I, IGF-II and IGFBP-3

The IGF-I, IGF-II and IGFBP-3 concentrations were measured by in-house radioimmunoassays (RIA). IGF-I and -II were measured after formic acid-acetone extrac-

tion. For the IGF-I RIA, cold IGF-II was added to decrease interference by binding proteins, and *vice versa*. The anti-IGF-I monoclonal antibody (BPL-M23, from Blood Products Ltd, Oxford Biotechnologies, Kidlington, Oxford, UK) was used at a final dilution of 1:20 000. The anti-IGF-II monoclonal antibody W5D2 (a gift from Prof. Anne White, Manchester, UK) was used at a final dilution of 1:250 000. The interassay percentage coefficient of variation (% cv) was 6.2% for IGF-I and 12.9% for IGF-II. IGFBP-3 was measured using the in-house antiserum (Cwyfan Hughes *et al.* 1995) at a final dilution of 1:20 000 (interassay % cv 5.1).

All of the methods used to characterise the myoblasts, their IGF-I and insulin receptor status, IGFBP profiles, and IGF-I and -II production were repeated a minimum of three times. Figures 1–6 are representative of the results obtained.

Growth factor effects on myotube protein synthesis and degradation

Protein synthesis and degradation were assessed using methods based on those described by Harper *et al.* (1987) and Roe *et al.* (1989). Briefly, responses to insulin (0, 0.5,

Figure 3 Desmin staining of muscle cells (magnification $\times 20$) after 7 days in differentiation medium showing stimulation of myoblast differentiation by IGF-I (A) and inhibition by FGF (B).

2.5, 5, 50 and 100 ng/ml) or IGF-I (0, 0.75, 7.5, 75, 150 and 300 ng/ml) were measured in myotubes. Long R³ IGF-I (LR³ IGF-I) was used to investigate the potential role of endogenously produced IGFBPs in modulating the response of the myotubes to IGF-I. Growth factors were prepared in serum-free medium immediately prior to use. L-[3,5-³H]Tyrosine (0.37 MBq; Amersham Life Science) in SFM or FM was added to the wells as described below. Muscle cells were grown to confluence and allowed to fuse for 7 days as described. Fibroblasts were used as a control. To assess synthesis, after two washes with PBS, the cells were incubated in SFM for 1 h before being dosed with peptide and tritiated tyrosine. After a 6-h incubation, (tyrosine is not metabolised to any detectable

extent over 72 h (Roe *et al.* 1989)), the media were discarded and all wells washed twice with PBS, followed by two washes with cold 5% trichloroacetic acid (TCA). Sodium hydroxide (0.5 M) was added to each well for 2 h at 37 °C to solubilise the cells. The protein concentration in the lysates was measured using the BCA protein assay. Protein synthesis was expressed as counts per minute tritiated tyrosine incorporated into cells over 6 h (counted using a Beckman LS 6500 Multipurpose Scintillation Counter) per mg protein. To determine protein degradation, for the final 48 h of the 7-day fusion period, the FM in the labelled wells was changed to include 0.37 MBq tritiated tyrosine per ml. After labelling, the cells were washed twice with PBS then incubated in SFM for

Figure 4 Rb dephosphorylation of muscle cells during 7 days in fusion medium. MCF-7 cell lysates are included as controls for phosphorylated (pRb) and non-phosphorylated Rb.

1 h before being dosed with IGF-I or insulin for 24 h. The media were collected and the wells were washed with cold PBS then cold 5% TCA; all the washings were collected and pooled with the harvested media. Ten percent TCA was added to each sample to bring its final concentration to 5%. Harvested samples were placed on ice for 20 min prior to centrifugation. The supernatants, acid precipitates and cell monolayers were all solubilised in 0.5 M NaOH. Protein degradation was expressed as follows: % protein degraded over 24 h = $100\% \times \text{radioactivity of medium amino acids} / (\text{radioactivity of medium amino acids} + \text{radioactivity of medium protein} + \text{radioactivity of cell protein})$. Each experiment was performed at least 3 times with wells in triplicate. In each experiment, a fourth unlabelled well was dosed as described, but in the absence of [^3H]tyrosine. CK activity of the cell lysates was measured in the unlabelled fourth wells, and the conditioned media from these wells was used to analyse IGF binding protein secretion.

Results

Characterisation of primary skeletal muscle cultures

Myoblast and myotube cultures were visualised by phase contrast light microscopy ($\times 20$ magnification). Although fibroblasts and myoblasts could not be thus distinguished, the progressive lining up and elongation of the myoblasts in FM and fusion to form multinucleate myotubes could be seen, as illustrated in Fig. 1. The cells were subsequently positively characterised by immunocytochemistry of the myotube cultures using desmin and titin (Fig. 2). Myotubes stain positively for both desmin and titin, whilst proliferating myoblasts stain positively for desmin in only 95% of cells (van der Ven *et al.* 1992). The fibroblast cultures did not stain with either desmin or titin (Fig. 2).

To substantiate these data, cells were cultured with IGF-I or FGF. Figure 3 illustrates the stimulation of myoblast differentiation by IGF-I and the inhibition of differentiation by FGF. Desmin staining of cytopins of myoblast cell suspensions showed that there was a mean of 97.6% muscle cells and 2.4% fibroblasts (standard deviation 2.76). *In vivo*, non-myogenic cells represent a substantial proportion (25–40%) of the nuclei of mature muscle (Roe *et al.* 1989), suggesting that these cultures are enriched for myogenic cells.

Morphological differentiation into myotubes was accompanied by a fourfold increase in biochemical differentiation as assessed by measuring the activity of the muscle-specific enzyme creatinine kinase. The median level of creatinine kinase activity measured in myotubes from 140 wells after seven days in fusion medium was 1851 U/g protein (25th–75th centiles 1228 and 2868 U/g). For comparison, Blau & Webster (1981) reported a creatinine kinase activity of 2515 U/g protein in their primary human skeletal myotube cultures after five days in fusion medium.

In order for skeletal myoblasts to terminally differentiate, they must arrest in G_0 and withdraw from the cell cycle (Gu *et al.* 1993). For this to occur, dephosphorylation and inactivation of the Rb protein is required (Schneider *et al.* 1994). We showed progressive Rb dephosphorylation during 7 days in FM, as illustrated in Fig. 4, coincidental with cells aligning and fusing, and with the increased CK activity.

Analysis of the IGF system in primary skeletal muscle cultures

Having successfully isolated and cultured primary human myoblasts which differentiated in low serum medium, we next assessed the IGF system. We found that both myoblasts and myotubes expressed insulin, IGF-I and

Figure 5 Immunoblots of skeletal muscle and fibroblast cell lysates showing (A) insulin, (B) IGF-I and (C) hybrid receptors. Description of lanes: B, myoblasts; T, myotubes; F, fibroblasts (200=200 µg protein loaded; 100=100 µg protein loaded).

insulin-IGF-I-hybrid receptors, as shown in Fig. 5, with the levels of all three receptor types increasing with time in differentiation medium. Optical density measurements comparing myotubes to myoblasts of the same passage and derived from the same subject showed a mean fold increase of 1.73 for the IGF-I receptor, 3.04 for the insulin receptor and 1.35 for the hybrid receptor. Insulin-IGF-I hybrid receptors have a similar affinity for IGF-I as the IGF-I receptor, but a 10-fold lower affinity for insulin than the insulin receptor (Soos *et al.* 1993), and appear to function like IGF-I receptors (Seely *et al.* 1995). They are formed in tissues expressing both IGF-I and insulin receptors (Federici *et al.* 1996, 1998). For positive comparison, fibroblasts also express both IGF-I and insulin receptors (Fig. 5).

Both myoblasts and myotubes produced a wide range of IGFBPs as illustrated in Fig. 6. Surprisingly, this included large amounts of intact IGFBP-3, which has not been shown to be produced by any of the other skeletal muscle cell lines examined to date (McCusker & Clemmons 1988, 1994, Tollefsen *et al.* 1989, Florini *et al.* 1995, Silverman *et al.* 1995). As the anti-IGFBP-3 antibody does not cross-react with bovine IGFBP-3 in FCS (see lane 6 of the IGFBP-3 immunoblot, Fig. 6A), all IGFBP-3 visualised by immunoblotting represented endogenous production by the cells. This was subsequently confirmed by IGFBP-3 radioimmunoassay of conditioned media. The anti-IGFBP-2 and anti-IGFBP-4 antibodies did cross-react with the binding proteins in the fetal calf serum, making it difficult to assess endogenous production by the

Figure 6 (A) Ligand blot and IGFBP-3 immunoblot of conditioned medium from primary human myoblasts, myotubes and fibroblasts. Lane 1, normal serum pool; lane 2, fibroblast serum-free medium; lane 3, fibroblast conditioned medium; lane 4, skeletal muscle serum-free medium; lane 5, skeletal muscle FM; lane 6, skeletal muscle GM; lane 7, myoblasts in GM (3 days); lane 8, myoblasts in serum-free medium (1 day); lane 9, myotubes in FM (3 days); lane 10, myotubes in serum-free medium (1 day).

myoblasts in growth medium, although endogenous production of both binding proteins by the differentiated myotubes in fusion medium was shown, as illustrated in Fig. 6B. In contrast to the adult muscle cells, fetal myoblasts produced much less IGFBP-3, IGFBP-2 and a smaller binding protein which was not IGFBP-4 (Fig. 6C). Again, for comparison, the primary human fibroblasts produced a more limited range of IGFBPs, one of which was IGFBP-3 (Fig. 6A).

Radioimmunoassay of myoblast and myotube conditioned media showed no significant endogenous production of IGF-I or IGF-II, above that found in the supplemented media alone (data not shown).

Growth factor effects on myotube protein synthesis and degradation

Having characterised the muscle cells with regard to phenotype and parameters of the IGF system, we wished to determine the functionality of the cells with respect to the physiological end points of protein synthesis and degradation. The dose-response curves showed that sig-

nificant anabolic responses to both IGF-I and insulin were seen at feasible physiological tissue concentrations of both peptides (Fig. 7A, B). The muscle cells tended to respond more efficiently to insulin than to IGF-I, suggesting that interference from the IGFBPs could be suppressing IGF-I action. We used LR³ IGF-I, a potent IGF-I analogue which has a very low affinity for the IGFBPs (Conlon *et al.* 1995), to test the hypothesis that the high levels of endogenous IGFBPs produced by the myotubes were reducing the activity of the exogenous IGF-I. Surprisingly, there were no significant differences in the cells' responses to physiological concentrations of IGF-I compared with LR³ IGF-I. There was considerable variability in the results obtained between experiments, possibly because of the use of cells derived from different subjects which have different degrees of differentiation capacity.

Discussion

We have established a primary adult human skeletal muscle cell culture system, which we have characterised

Figure 6 (B) Ligand blot and immunoblots for IGFBP-2 and IGFBP-4 of conditioned medium from primary human myoblasts and myotubes. Lane 1, normal serum pool; lane 2, skeletal muscle GM; lane 3, myoblasts in GM (3 days); lane 4, myotubes in FM (2 days); lane 5, myotubes in FM (4 days); lane 6, skeletal muscle FM.

immunocytochemically by desmin and titin staining and biochemically by measuring creatinine kinase activity. Terminal differentiation and myotube formation was associated with Rb dephosphorylation. FGF stimulated replication of the myoblasts but inhibited differentiation, whilst IGF-I stimulated both replication and differentiation into myotubes. We therefore have access to a clinically relevant model in which to study the pathophysiology of muscle wasting.

With respect to the IGF system, we have shown that the cells express insulin, IGF-I and insulin-IGF-I hybrid

receptors, with increased levels of all three types of receptor in myotubes compared with myoblasts. In the BC3H-1, C2 and L6 skeletal muscle cell lines, differentiation is associated with increased insulin but decreased IGF-I receptors levels (Beguino *et al.* 1985, Brunetti *et al.* 1989, Rosenthal *et al.* 1991) although Tollefsen *et al.* (1989) reported a transient increase in ^{125}I -IGF-I binding 72 h after the induction of differentiation in C2 cells. In primary adult human skeletal muscle cells, Shimizu *et al.* (1986) found that ^{125}I -insulin binding increased on differentiation (being below their limit of detection in

Figure 6 (C) Ligand blot and immunoblots for IGFBP-3 and IGFBP-2 of conditioned medium from human fetal myoblasts. Lane 1, normal serum pool; lane 2, fetal myoblasts in serum-free medium (1 day).

myoblasts), whilst there was no difference in ^{125}I -IGF-I binding between myoblasts and myotubes. Our primary cultures produced a wide range of high affinity IGFBPs, including IGFBP-2, -4 and abundant IGFBP-3. The fetal myoblast IGFBP profile included much less IGFBP-3, IGFBP-2 and no IGFBP-4. Both profiles contrast with rat L6 muscle cells, which produce IGFBP-4, -5 and -6 (McCusker & Clemmons 1988, 1994, Silverman *et al.* 1995), and mouse C2 cells, which only produce IGFBP-5 (Tollefsen *et al.* 1989, Florini *et al.* 1995). When fibroblasts are cultured with transforming growth factor- β , they enter cell cycle arrest. A postulated mechanism is the stimulation of increased production of IGFBP-3 (Kim *et al.* 1997). The high levels of endogenous IGFBP-3 production by adult human myoblasts could trigger premature cell senescence *in vitro*, and may explain some of the difficulties experienced by ourselves and others in culturing these cells. It may also inhibit aberrant replication of adult skeletal muscle cells *in vivo*. Our primary myoblast and myotube cultures did not produce significant amounts of IGF-I or IGF-II peptide.

We found that both insulin and IGF-I had anabolic effects on protein metabolism at physiological concentrations. Insulin was more potent than IGF-I, with lower concentrations required to achieve similar maximal responses. Others have found IGF-I to have more potent protein anabolic effects on myotubes than insulin, the latter being effective only at supraphysiological concentrations when it may have been acting as an IGF-I analogue (Shimizu *et al.* 1986, Harper *et al.* 1987, Roe *et al.* 1989). There are several possible explanations for the differences between our physiological findings and those of others. Our use of recombinant human insulin as opposed

to bovine or porcine insulin may explain the relatively high potency of insulin we observed. Alternatively, it may reflect different proportions of IGF-I, insulin and hybrid receptors in the different models. Finally, our data may provide key evidence of the differential effects of insulin and IGF-I which have been postulated, with insulin being a more potent anabolic agent for skeletal muscle protein metabolism, and IGF-I acting predominantly to stimulate muscle cell proliferation and differentiation. Unexpectedly, the equivalence of the responses to IGF-I and LR³ IGF-I suggested that the wide range of IGFBPs secreted by the cells did not affect the response to exogenous IGF-I with regard to protein anabolism.

There are important differences in the IGF-I receptor status, IGFBP-3 production and the response to insulin of our primary adult human skeletal muscle cells, compared with the results of other investigators using skeletal muscle cell lines and often working with undifferentiated myoblasts rather than differentiated myotubes. This highlights the importance of developing a model which is as physiologically relevant as possible within the constraints of a cell culture system. The IGF system plays a pivotal role in the regulation of skeletal muscle cell cycling and intermediary metabolism in health and disease. We have fully characterised our model with reference to muscle cell status and capacity to differentiate *in vitro*. We have also assessed the IGF receptor status, binding protein and peptide secretion, and have obtained preliminary results related to protein metabolism indicating both the functionality of the cells and an increased sensitivity to the anabolic effects of insulin compared with IGF-I.

We are now in a position to address more complex questions regarding the pathophysiology of muscle wasting

Figure 7 Protein synthesis (A) and degradation (B) of skeletal muscle cells in response to IGF-I (◆) and insulin (□). Experiments were performed with wells in triplicate (the error bars represent 1 S.D.), and each experiment was repeated at least 3 times.

associated with many chronic severe illnesses. These include interactions between the growth hormone–IGF system, other hormones (such as insulin, cortisol and leptin), and the inflammatory cytokines (tumour necrosis factor- α and interleukins-1 and -6) in the regulation of intermediary metabolism, proliferation, differentiation and apoptosis in skeletal muscle cells. A slight imbalance in any of these processes could influence the equilibrium between muscle maintenance and muscle destruction, resulting in a marked loss of lean body mass. We plan to investigate whether muscle cells grown from patients with cancer

maintain a ‘cachectic phenotype’ *in vitro* with respect to protein metabolism, analogous to the *in vitro* insulin resistance of muscle cell cultures derived from patients with Type II diabetes and obesity (Thompson *et al.* 1996). This would suggest that there are intrinsic abnormalities of the muscle cells in addition to abnormalities in their hormonal and cytokine milieu, which, if identified, could potentially be manipulated and reversed. In the future, such research may lead to the development of novel therapeutic interventions to target the important clinical problem of cachexia, for which at present no effective treatment is available.

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