# Insulin-like growth factor (IGF-I) induces myotube hypertrophy associated with an increase in anaerobic glycolysis in a clonal skeletal-muscle cell model

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Insulin-like growth factor-I (IGF-I) is an important autocrine/paracrine mediator of skeletal-muscle growth and development. To develop a definitive cultured cell model of skeletal-muscle hypertrophy, C2C12 cells were stably transfected with IGF-I and clonal lines developed and evaluated. Quantitative morphometric analysis showed that IGF-I-transfected myotubes had a larger area ($2381 \pm 60\ \mu m^2$ versus $1429 \pm 39\ \mu m^2$; $P < 0.0001$) and a greater maximum width ($21.4 \pm 0.6\ \mu m$ versus $13.9 \pm 0.3\ \mu m$; $P < 0.0001$) than control C2C12 myotubes, independent of the number of cell nuclei per myotube. IGF-I-transfected myotubes had higher levels of protein synthesis but no difference in DNA synthesis when compared with control myotubes, indicating the development of hypertrophy rather than hyperplasia. Both lactate dehydrogenase and alanine aminotransferase activities were increased (3- and 5-fold respectively), and total lactate levels were higher (2.3-fold) in IGF-I-transfected compared with control myotubes, indicating an increase in anaerobic glycolysis in the hypertrophied myotubes. However, expression of genes involved in skeletal-muscle growth or hypertrophy *in vivo*, e.g. myocyte nuclear factor and myostatin, was not altered in the IGF-I myotubes. Finally, myotube hypertrophy could also be induced by treatment of C2C12 cells with recombinant IGF-I or by growing C2C12 cells in conditioned media from IGF-I-transfected cells. This quantitative model should be uniquely useful for elucidating the molecular mechanisms of skeletal-muscle hypertrophy.

Key words: development, growth, metabolism, quantitative.

## INTRODUCTION

Cell proliferation (mitogenesis) and differentiation (myogenesis) during muscle development are complex processes, with the majority of mitogens stimulating skeletal-muscle proliferation but inhibiting differentiation. Insulin-like growth factor-I (IGF-I) is therefore unique among growth factors as it stimulates *both* proliferation and differentiation of skeletal-muscle cells in culture [1–7]. In normal cell division, most cell lines require IGF-I, i.e. it is an important requirement for cells traversing the $G_1$ phase of the cell cycle. In addition, IGF-I is an essential requirement for the synthesis of proteins and nucleic acids, and for glucose metabolism [8,9]. In skeletal-muscle cells, IGF-I initially promotes proliferation by enhancing the expression of intracellular mediators, e.g. cyclin D and c-*fos*, and by suppressing other mediators of the myogenic response, e.g. myogenin [10]. This process is followed by a strong myogenic response during which myoblasts withdraw from the cell cycle and express muscle-specific transcription factors, such as myogenin, ultimately leading to fusion of myoblasts to form multinucleated myotubes [11,12]. These stimulatory effects of IGF-I involve specific binding to high-affinity membrane-associated receptors that are tyrosine kinases [13].

Evidence that IGF-I is an important autocrine/paracrine mediator of skeletal-muscle growth and development *in vivo* is the finding that overexpression of IGF-I in transgenic mice induced myotube formation in developing embryos, stretch-induced myofibre hypertrophy and muscle regeneration following injury, as well as increased body growth [14–16]. Moreover, IGF-I-deficient mice are significantly smaller than their normal litter mates, have severe muscular dystrophy, and the majority ($> 95\%$) die at, or immediately after, birth [17].

The development of myofibre hypertrophy with IGF-I overexpression *in vivo* has not been demonstrated clearly in an *in vitro* model. Several *in vitro* studies have suggested qualitatively that growth factors added to or transfected into skeletal-muscle cells result in myotubes that 'appear larger' morphologically [18–20]. These studies have shown that IGF-I increases contractile-protein mRNA, myogenic basic helix-loop-helix factor and myotube formation. Interleukin-15, a potential anabolic agent, has also been shown to increase myosin heavy-chain accumulation and result in myotubes that 'appear larger' [20]. Thus no definitive cultured cell model is available currently to evaluate the molecular and biochemical mechanisms underlying skeletal-muscle hypertrophy.

The present study was undertaken to develop a quantitative *in vitro* model of skeletal-muscle cell hypertrophy using the C2C12 cell line. This cell line was selected since it is a pure, immortalized mouse satellite cell line, which is morphologically similar to primary satellite-cell cultures [21–23]. Following the loss of skeletal muscle through disease or injury, new muscle fibres are formed *in vivo* by proliferation, fusion and maturation of satellite cells; responses that can be induced readily in C2C12 cells in culture. Moreover, unlike other muscle cell lines, e.g. L6 cells, C2C12 cells have an unrestricted profile of myogenic-factor expression, including myf-5, myogenin, MRF (myogenic regulatory factor)-4 and MyoD, and also express both IGF-I and IGF-II. Finally, in contrast with the receptors for other growth

Abbreviations used: IGF-I, insulin-like growth factor-I; IRES, internal ribosome-entry site; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; $K_s$, fractional synthesis rate; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; MNF, myocyte nuclear factor; MRF, myogenic regulatory factor.

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factors, expression of IGF-I receptors is not down-regulated following differentiation of C2C12 cells into myotubes [24,25]. Using quantitative morphometric analysis together with evaluations of protein and DNA synthesis, we demonstrate the development of myotube hypertrophy in both clonal IGF-I-transfected C2C12 cells and in normal C2C12 cells treated exogenously with IGF-I. Hypertrophy in these C2C12 myotubes is associated with an increase in anaerobic glycolysis, as indicated by an elevation both in the activity of glycolytic enzymes and in the final product of anaerobic glycolysis, lactate. Availability of this clonal cell model should be uniquely useful for understanding the molecular events involved in skeletal-muscle hypertrophy.

## MATERIALS AND METHODS

### Development of IGF-I construct

An elongation factor-1 promoter green fluorescent protein (GFP) 20 expression vector carrying an internal ribosome-entry site (IRES) was used (plasmid size 8.0 kb). The GFP20 was designed by Dr. S. Aota (BERI, Osaka, Japan) and contains the mutations S65A, V68L, S72A, F99S, M153T and V163A. This mutant form of GFP fluoresces more intensely than the wild-type protein. A *Bam*HI fragment containing the IGF-I cDNA was isolated from a pUC12 plasmid (kindly provided by Ms. C. Lee) and ligated into the GFP vector at a unique *Bam*HI cloning site. Correct orientation of the insert was confirmed by sequencing and restriction-enzyme analysis. The resulting expression cassette is shown in Figure 1 (upper panel).

### Cell lines, transfections and culture conditions

The murine myoblast line C2C12 was utilized [21,22] to generate stable transfectants. This was achieved by co-transfection of C2C12 myoblasts with the IGF-I/IRES/GFP20 plasmid (IGF-I-transfected cells) or the same plasmid without the IGF-I cDNA (control cells) with a plasmid, pcDNA3, encoding the neomycin-

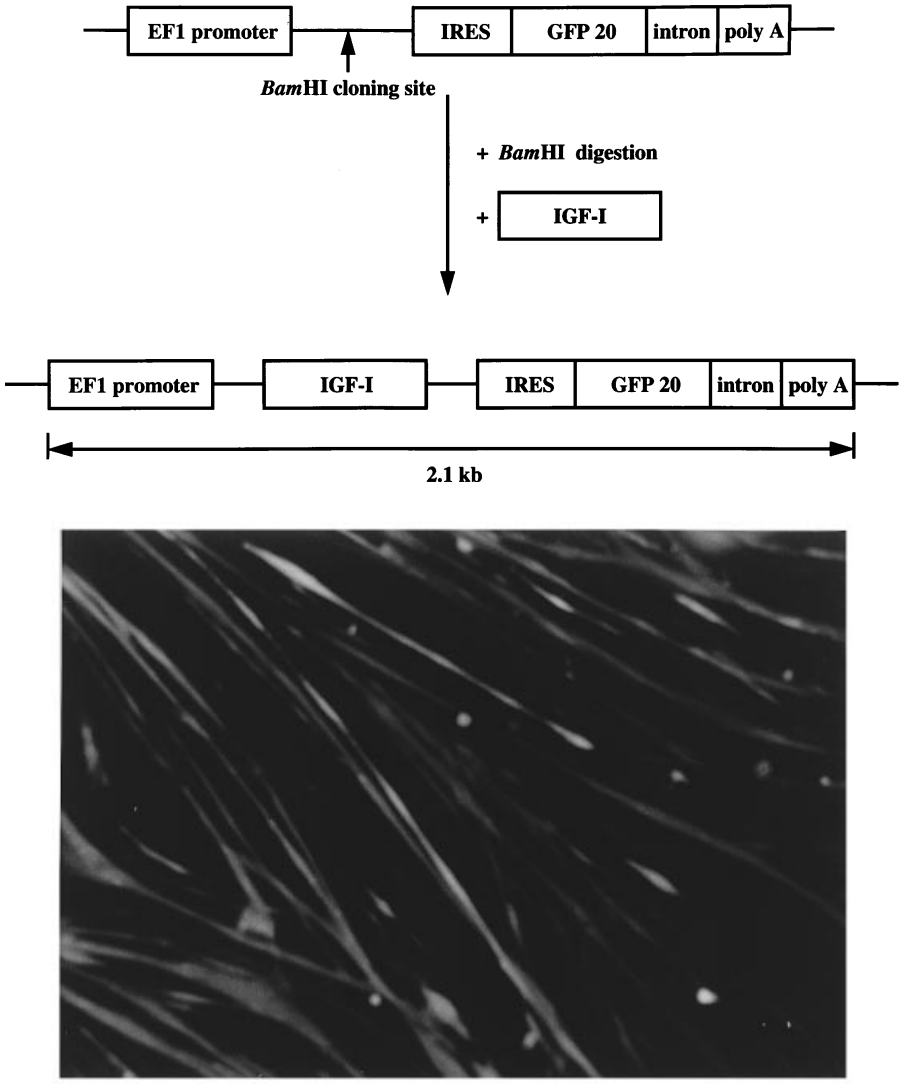


**Figure 1 IGF-I stably transfected C2C12 myotubes expressing GFP**

The upper panel shows the expression cassette used to stably transfect C2C12 cells. The IGF-I fragment was inserted into a unique *Bam*HI cloning site in a bicistronic vector containing an elongation factor-1 (EF1) promoter, an IRES, a GFP20 marker gene and an intron/poly-A tail. The lower panel shows C2C12 myotubes stably transfected with the construct in the upper panel, expressing both GFP20 and IGF-I. Fluorescence was observed using FITC-equipped blue-light microscopy.

resistance gene. Liposome-mediated transfection was performed. Briefly, myoblasts were grown in 100 mm plates to 50% confluence. Cells were then washed with PBS (0.01 M $NaPO_4$/140 mM NaCl/0.25 mM KCl). DNA (10 $\mu$g of pGFP/IGF-I and 1 $\mu$g of pcDNA3), 75 $\mu$l of Lipofectamine (Life Technologies, Grand Island, NY, U.S.A.) and 1.6 ml of serum-free medium were incubated at room temperature for 45 min and then added to the plates for 5 h before replacement with normal growth medium, i.e. Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum, penicillin/streptomycin (5000 units/5000 $\mu$g per ml) and 0.5% chick embryo extract. Colonies were then selected with Geneticin (G418 sulphate; Life Technologies) and collected from the plates using 8 mm cloning cylinders. Each clone was then grown separately. Stable transfectant myoblasts were maintained in the 20% growth medium containing Geneticin (200 $\mu$g/ml). To induce myogenic differentiation, the medium was changed to DMEM containing 2% horse serum and 200 $\mu$g/ml Geneticin. GFP20 fluorescence was assessed using a binocular microscope equipped for fluorescence with a FITC filter (Zeiss Axiolab, Oberkochen, Germany).

### Administration of exogenous IGF-I

In addition to the studies of stably transfected cell lines expressing IGF-I, human recombinant IGF-I (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added to the medium of normal C2C12 cells at concentrations of 25 ng/ml and 250 ng/ml to study the effects of exogenously-treated C2C12 cells. IGF-I was added at various times during either proliferation or post-fusion. To study the paracrine effects of IGF-I, normal C2C12 cells were grown exclusively using the medium harvested from stably transfected IGF-I cells. Every 12 h, the medium from the IGF-I-transfected cells was transferred to the normal C2C12 cells. Differentiation was allowed to occur and the resulting myotubes analysed.

### [$^{35}$S]Cysteine/methionine labelling

Myoblasts were labelled with 0.5 mCi of [$^{35}$S]cysteine (30%)/methionine (70%) (Amersham Life Science, Arlington, IL, U.S.A.) for 12 h as previously described [26] and used for immunoprecipitation. In brief, cells were rinsed with 1 × PBS and both the cells and supernatant were treated with RIPA buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% SDS, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 1 mM EDTA. Cells were centrifuged for 5 min at 8000 ***g*** at 4 °C. To both the cell lysate and supernatant, 1 $\mu$g of anti-IGF-I antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added and the samples incubated on ice for 60 min. The immune complexes were incubated with a 10% slurry of Protein A–agarose for 45 min on ice. The immunoprecipitates were then collected, washed three times with RIPA buffer, heated to 100 °C in SDS-gel loading buffer, and fractionated on an SDS/10% polyacrylamide gel. Gels were fixed with 0.1 M sodium salicylate, dried and exposed to Kodak (BiomaxMR) film at room temperature.

### Northern-blot analysis

Total cellular RNA was isolated from C2C12 cells grown on 100 mm tissue-culture plates as previously described [27]. RNA was extracted using 2 ml of solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M $\beta$-mercaptoethanol and 0.2 M sodium acetate. Chloroform (200 $\mu$l) was added and the samples left on ice for 10 min. Samples were then centrifuged at 8000 ***g*** for 10 min, the supernatant collected, added to an equal volume of propan-2-ol and left on ice for a further 10 min. Samples were centrifuged at 8000 ***g*** for 10 min at 4 °C. The RNA pellet was dried and reconstituted in 200 $\mu$l of diethyl pyrocarbonate-treated water. Approximately equal amounts of RNA were loaded in each lane of a 1% agarose gel containing 6% formaldehyde and electrophoresed in a 1 × Mops buffer. RNA was transferred to a nitrocellulose membrane (Immobilin-NC; Millipore, Bedford, MA, U.S.A.) and cross-linked with UV light (UV Stratlinker 2400; Stratagene, La Jolla, CA, U.S.A). Prehybridization (2 h) and hybridization (overnight) were performed at 42 °C in 50% formamide/3 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/50 mM Hepes (pH 7.0) and 1 × Denhardt's buffer. Probes were prepared from full-length IGF-I cDNA fragments using a random primer DNA-labelling kit (Prim-a-gene Labelling Kit; Promega, Madison, WI, U.S.A.) to incorporate 50 $\mu$Ci of [$\alpha$-$^{32}$P]dATP (DuPont, Boston, MA, U.S.A.). Ethidium bromide staining and probing with glyceraldehyde-3-phosphate dehydrogenase was used to quantify RNA loading. mRNA quantification was performed using image-analysis software (Personal Densitometer SI and Phosphorimager 445 SI; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

### DNA- and protein-synthesis assays

DNA synthesis was measured by [$^3$H]thymidine incorporation. [$^3$H]Thymidine (5.0 $\mu$Ci; Amersham) was added to cells in a 24-well tissue-culture plate, and placed in a 37 °C incubator for 24 h. Cells were washed twice with 1 × PBS and then stained with 5% Giemsa stain. The number of nuclei per high-power field was determined. Five separate randomly selected fields were counted to quantificate the numbers of nuclei in the myoblast and myotube samples. Cells were again washed with 1 × PBS and 10% trichloroacetic acid added to each well for 10 min. Cells were collected and [$^3$H]thymidine uptake measured in a scintillation counter. Protein synthesis was measured by L-[2,6-$^3$H]phenylalanine (Amersham) incorporation and calculation of fractional synthesis rates [28]. L-[2,6-$^3$H]Phenylalanine (5.0 $\mu$Ci) was added to cells in 6-well tissue-culture plates flooded with 0.5 mM unlabelled phenylalanine. Cells were placed in a 37 °C incubator for 2 h. Cells were then washed three times with 1 × PBS, collected and homogenized in 10% trichloroacetic acid with a Polytron. The precipitated proteins were then pelleted by centrifugation and the supernatants harvested for the determination of phenylalanine concentration and radioactivity. The protein pellets were washed three times by addition of 10% trichloroacetic acid and then centrifugation, with the supernatants from each wash being discarded. The final pellets were retained for determination of phenylalanine concentration and radioactivity. Phenylalanine concentrations in aliquots of the supernatants harvested above and in the final pellets were determined by amino acid analysis (Australian Proteome Analysis Facility, Sydney, Australia) after initial hydrolysis with 6 M hydrochloric acid at 115 °C for 24 h. The radioactivity of the supernatants and pellets was determined by $\beta$-scintillation spectrometry at an efficiency of 45%. The fractional rate of protein synthesis ($K_s$), as a percentage/day, was then calculated using the following formula:

$$K_s = [S_b/S_a \times 1440/t] \times 100$$

where $S_b$ is the specific radioactivity of protein-bound phenylalanine, $S_a$ the specific radioactivity of free phenylalanine, and $t$ is the time of labelling (in min).

### Morphological analysis

Myotube area and width were calculated using the Leica Q500 MC image-analysis software. Myotube plates were fixed with 10% methanol and stained with 5% Giemsa stain. Individual myotubes were planimetered to calculate total myotube area and maximum myotube width. A total of 100 myotubes were analysed from 10 randomly selected microscopic fields for each study group.

### Glycolytic enzyme assays and determination of lactate levels

Lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) enzyme activities, and determination of lactate levels, were performed using standard calorimetric methods (Sigma Diagnostics procedure nos. 500, 505 and 735; St. Louis, MO, U.S.A.). Briefly, the LDH assay involved adding 1 ml of pyruvate substrate to an NADH-containing vial (1 mg/ml) and incubating at 37 °C for 5 min. The cell extract (100 $\mu$l) was added, mixed gently and incubated at 37 °C for 30 min. To each vial, 1 ml of Sigma Color Reagent (Sigma Diagnostics) was added, mixed and allowed to stand at room temperature for 20 min. Then, 10 ml of 0.4 M sodium hydroxide was added and, after 5 min, absorbance at 525 nm was measured. The ALT assay involved addition of 200 $\mu$l of the cell extract to a test tube containing 1 ml of alanine/$\alpha$-ketoglutarate substrate (Sigma Diagnostics). This was gently mixed and incubated at 37 °C for 30 min. To each test tube, 1 ml of Sigma Color Reagent was added and the remainder of the assay was as for the LDH assay. Absorbance for ALT was measured at 505 nm. In measuring lactate levels, 1 ml of lactate reagent solution (Sigma Diagnostics) was added to 100 $\mu$l of the cell extract and incubated at room temperature for 10 min. Absorbance was recorded at 540 nm. In all three assays, standard calibration curves were derived for the Perkin-Elmer Lambda Bio UV/Vis spectrometer used in these studies. All assays were performed in triplicate on at least two different extracts.

### Construction of myocyte nuclear factor (MNF) probes

Probes for $\alpha$- and $\beta$-MNF were made as previously described [29]. In brief, amplified products were produced from C2C12 RNA by reverse transcriptase-PCR with primers specific for $\alpha$- and $\beta$-MNF. The 5′ primer, TACTTCATCAAAGTCCCTCGGTC, was common to both, whereas the 3′ primers, GTACTCTGGAACAGAGGCTAACTT and GTGCGCGCGCGCATGTGGGGC, were specific for $\alpha$- and $\beta$-MNF, respectively. The resulting products were 307 and 216 nucleotides long, respectively. Probes were then made from these cDNA fragments using the 5′-random-primer DNA-labelling kit and Northern-blot analysis was performed as described above.

### Statistical analysis

Comparisons between study groups were made using paired or unpaired *t*-tests, with a $P$ value of $< 0.05$ being considered statistically significant. All data are expressed as means ± S.E.M.

## RESULTS

### Stably transfected IGF-I myoblasts have increased IGF-I mRNA and protein expression

From a total of 10 IGF-I-positive C2C12 clones, three were used to study the effects of IGF-I on myoblast proliferation and differentiation. Figure 1 (lower panel) demonstrates GFP-mediated fluorescence in stably transfected C2C12 myotubes.

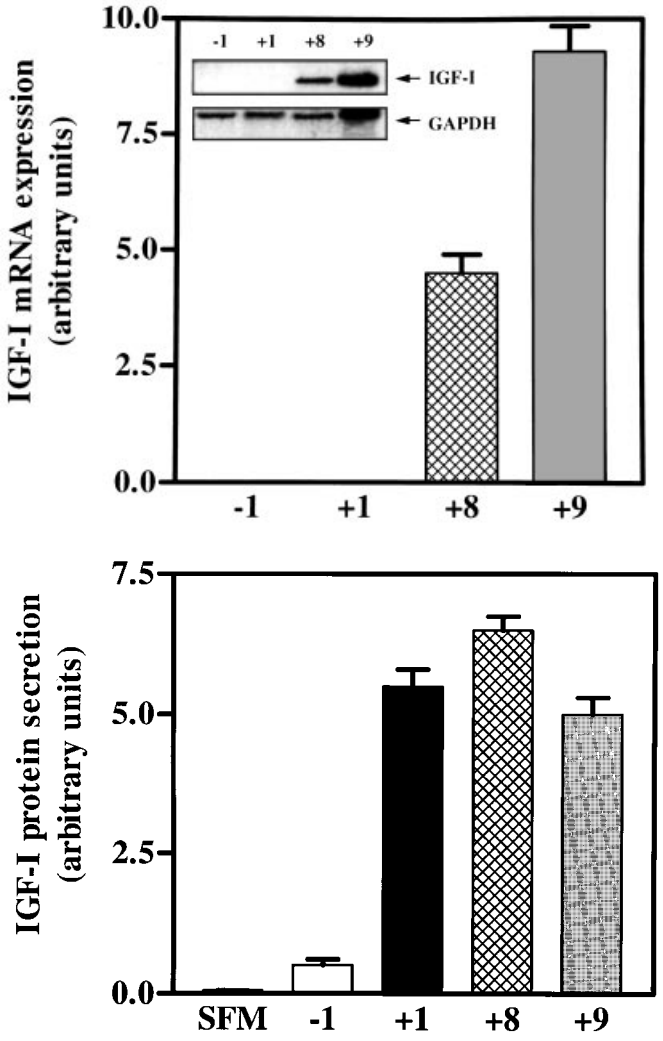


**Figure 2 Stably transfected IGF-I myoblasts have increased IGF-I mRNA and protein**

The upper panel shows variable amounts of IGF-I mRNA in three different IGF-I-positive clones (+1, +8, +9). No endogenous IGF-I mRNA was detected in the normal control C2C12 cells (−1). +, Positive IGF-I DNA control. The densitometry analysis of the IGF-I mRNA Northern-blot analysis (inset) allows correction for differences in loading [based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels]. The lower panel shows the amount of IGF-I protein secretion based on [$^{35}$S]cysteine/methionine labelling, immunoprecipitation and densitometric analysis performed as described in Materials and methods. IGF-I protein secretion is significantly elevated in the three different IGF-I-positive clones. Labelling of a small amount of endogenous IGF-I secretion is seen in the control cells. SFM indicates amount of IGF-I in serum-free medium. Data are expressed as means ± S.E.M.

Following complete differentiation and fusion, myotubes were able to survive for approximately 14 days before there was microscopic evidence of myotube degeneration and cell death. There was no difference in survival between IGF-I-transfected and untreated myotubes. Although IGF-I mRNA expression differed somewhat between three different IGF-I positive clones and was not detectable in control cells (Figure 2, upper panel), [$^{35}$S]cysteine/methionine labelling and immunoprecipitation studies demonstrated similar significantly elevated levels of secreted IGF-I protein in the medium of IGF-I-transfected cells compared with controls (Figure 2, lower panel).

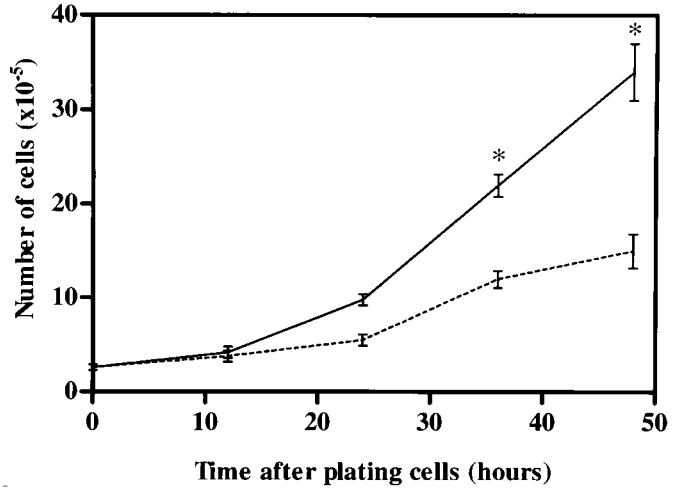


**Figure 3 IGF-I increases proliferation in C2C12 myoblasts**

The time-dependent increase in the number of myoblasts of control (-----) and IGF-I-transfected (——) cells is shown (*, $P < 0.01$). At 48 h, there is an approximately 3-fold increase in the number of myoblasts transfected with IGF-I compared with control myoblasts. Data are expressed as means ± S.E.M. for three independent experiments in three different clonal lines.

**Table 1 Changes in DNA and protein synthesis in clonal IGF-I-transfected cells**

Data are means ± S.E.M. for three independent experiments in two different IGF-I clonal lines. *, $P < 0.01$ versus respective controls.

| | $10^{-4} \times$ [$^3$H]Thymidine incorporation (d.p.m.) | $K_s$ (%/day) |
|---|---|---|
| Control myoblasts | 21.9 ± 2.1 | 35 ± 7 |
| IGF-I myoblasts | 36.2 ± 7.5* | 82 ± 12* |
| Control myotubes | 33.9 ± 3.5 | 27 ± 3 |
| IGF-I myotubes | 34.9 ± 1.1 | 73 ± 11* |

## IGF-I increases proliferation and stimulates DNA synthesis in myoblasts but not myotubes

Normal and IGF-I-transfected myoblasts maintained in DMEM plus 20 % fetal calf serum were counted at 0, 12, 24, 36 and 48 h after plating. By 48 h, confluency had increased to approximately 80 %. There was a significant increase in the number of proliferating cells in the IGF-I-transfected myoblasts (Figure 3). Consistent with this IGF-I-stimulated increase in proliferation, DNA synthesis was also increased in the IGF-I-transfected myoblasts, as evidenced by a greater increase in [$^3$H]thymidine uptake in these cells compared with control myoblasts (Table 1). However, consistent with cessation of proliferation and the development of hypertrophy, [$^3$H]thymidine uptake, normalized for the number of nuclei per myotube, was not increased in IGF-I-transfected myotubes as compared with control myotubes (Table 1). In these myotube studies, no significant differences were observed in [$^3$H]thymidine uptake between control cells pre-treated with cytosine arabinoside (to eliminate any undifferentiated cells post-fusion) and untreated control cells and, therefore, cytosine arabinoside was not used in ensuing studies.

**Table 2 Morphometric changes in clonal IGF-I-transfected myotubes**

Data are means ± S.E.M. for three independent experiments in three different IGF-I clonal lines. All values are significantly higher in the stably transfected IGF-I myotubes than in the respective controls ($P < 0.0001$).

| | Myotube area ($\mu$m$^2$) | Myotube width ($\mu$m) |
|---|---|---|
| Two-nuclei | | |
| Control | 1337 ± 33 | 13.3 ± 0.3 |
| IGF-I | 2117 ± 55 | 19.8 ± 0.9 |
| Three-nuclei | | |
| Control | 1682 ± 54 | 15.3 ± 0.5 |
| IGF-I | 2726 ± 84 | 23.7 ± 0.6 |
| Four-nuclei | | |
| Control | 2247 ± 69 | 21.0 ± 0.9 |
| IGF-I | 3311 ± 94 | 30.0 ± 1.6 |

## IGF-I increases fractional protein synthesis in both myoblasts and myotubes

As shown in Table 1, the $K_s$ value was significantly greater in both IGF-I-transfected myoblasts and myotubes compared with their respective controls. Since [$^3$H]thymidine uptake was increased in IGF-I myoblasts but not myotubes, the increase in protein synthesis in the IGF-I myoblasts was the result of IGF-I-induced proliferation in myoblasts. In contrast, in IGF-I-transfected myotubes, the increased protein synthesis in the absence of an increase in DNA synthesis is consistent with the induction of hypertrophy.

## IGF-I induces myotube hypertrophy independently of the number of fused cells

Using quantitative image analysis, myotube hypertrophy was evident in the IGF-I-transfected myotubes based on both total myotube area and myotube width. Thus IGF-I-transfected myotubes had a larger cell area (2381 ± 60 $\mu$m$^2$ versus 1429 ± 39 $\mu$m$^2$; $P < 0.0001$) and a greater maximum myotube width (21.4 ± 0.59 $\mu$m versus 13.9 ± 0.33 $\mu$m; $P < 0.0001$) than control C2C12 myotubes. As a potential explanation for this hypertrophic response may be an increase in the number of fused cells per myotube, a sub-analysis was performed to compare myotubes with the same number of nuclei in the two cell groups. The increases in myotube area and width were again demonstrated in the comparison of bi-, tri- and tetra-nucleated myotubes (Table 2). Whereas a significant proportion of myotubes contained more than four nuclei, these were not included in the analyses, as counting the number of nuclei (which were often overlapping) became difficult to assess accurately.

## Exogenous IGF-I induces myotube hypertrophy when added to normal C2C12 cells during proliferation but not post-differentiation

IGF-I acts as both an autocrine and paracrine growth factor [30]. The fact that IGF-I is secreted and can thus act in a paracrine or hormonal manner may enhance the efficiency of muscle-mediated IGF-I gene therapy by allowing IGF-I to stimulate untransfected cells or distant organs and tissues. To study the paracrine effects

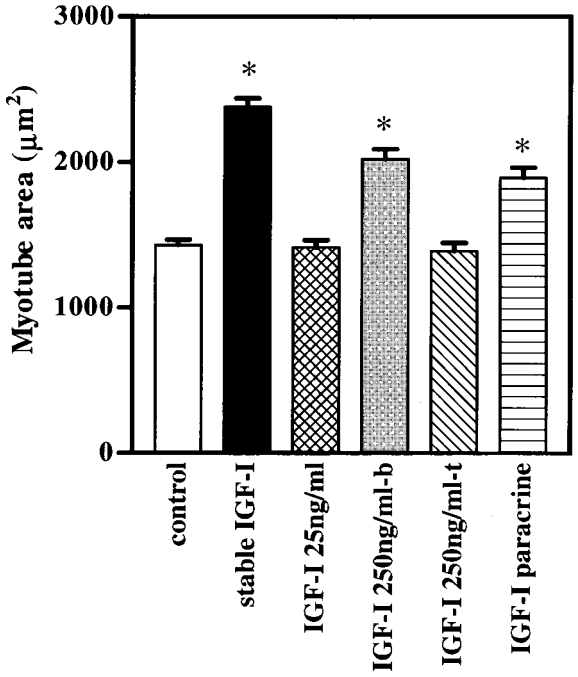


**Figure 4 Exogenous IGF-I induces myotube hypertrophy in normal C2C12 myoblasts but not myotubes**

Recombinant IGF-I added to the medium of normal myoblasts resulted in dose-dependent myotube hypertrophy. Hypertrophy was not seen in myoblasts treated with 25 ng/ml of recombinant IGF-I but was seen in myoblasts treated with 250 ng/ml (IGF-I 250 ng/ml-b; *, $P < 0.001$ versus control). Myotube hypertrophy also resulted when normal C2C12 cells were grown in medium collected from stably transfected IGF-I cells (IGF-I paracrine; *, $P < 0.001$), thus demonstrating the paracrine effects of IGF-I. Recombinant IGF-I added to the medium for 48 h, at either 48 h or 7 days post-differentiation, did not induce a hypertrophic response (IGF-I 250 ng/ml-t). Data are expressed as means ± S.E.M. for three independent experiments in three different clonal lines.

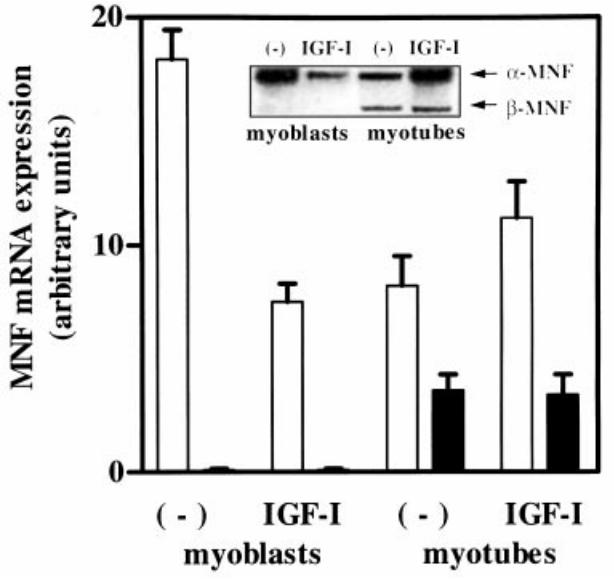


**Figure 5 MNF expression in IGF-I-transfected cells**

Significant levels of $\alpha$-MNF (white bars) mRNA expression were observed in both control (—) and IGF-I-transfected myoblasts and myotubes. Although the $\alpha$-MNF mRNA levels were lower in the IGF-I-transfected myoblasts compared with control myoblasts ($P < 0.01$), the difference between the IGF-I-transfected and control myotubes was not significant. $\beta$-MNF (black bars) mRNA expression was not detectable during the myoblast stage in control or IGF-I-transfected cells. Post-differentiation, low levels of $\beta$-MNF expression were observed in myotubes although the levels of expression did not differ significantly between control and IGF-I-transfected cells. All mRNA data were corrected for loading differences based on ethidium bromide staining. Data are means ± S.E.M. for two independent experiments.

of IGF-I, normal C2C12 myoblasts were treated with either recombinant IGF-I (25 ng/ml and 250 ng/ml) or the medium harvested from IGF-I-transfected cells. Figure 4 shows the comparison of the mean myotube area in these treated cells. No hypertrophy was seen in the normal C2C12 myoblasts treated with a low dose (25 ng/ml) of recombinant IGF-I. However, significant myotube hypertrophy was observed in normal C2C12 myoblasts treated with a higher dose (250 ng/ml) of recombinant IGF-I, and in normal C2C12 myoblasts treated with the medium of IGF-I-transfected cells. This conditioned medium had an IGF-I level (269 ng/ml) in the equivalent range to that of the recombinant IGF-I studies that induced myotube hypertrophy, suggesting that this paracrine effect was due to the secreted IGF-I in the conditioned medium. Addition of recombinant IGF-I (250 ng/ml) to normal C2C12 cells at 48 h and 7 days post-differentiation did not result in the induction of hypertrophy. These results demonstrate the paracrine role of IGF-I in mediating the process of myotube hypertrophy and the inability of IGF-I to induce hypertrophy post-differentiation.

### Myotube hypertrophy is associated with an increase in both glycolytic enzyme activities and lactate levels

In the myoblast stage, there was no difference in LDH or ALT (Table 3) activities between IGF-I-transfected and control cells. In contrast, there was a significant increase in both LDH (3-fold) and ALT (5.5-fold) activities in the IGF-I-transfected myotubes

**Table 3 Glycolytic enzyme activities and lactate levels in clonal IGF-I-transfected cells**

Data are means ± S.E.M. for three independent experiments in two different IGF-I clonal lines. *, $P < 0.01$ versus control myotubes. BB units, Berger–Broida units; SF units, Sigma–Frankel units.

| | $10^{-3} \times$ LDH activity (BB units/mg) | $10^{-3} \times$ ALT activity (SF units/mg) | Lactate level (mg/l) |
|---|---|---|---|
| Control myoblasts | 4.8 ± 0.4 | 0.2 ± 0.05 | 64 ± 3.0 |
| IGF-I myoblasts | 5.9 ± 0.3 | 0.2 ± 0.05 | 85 ± 5.0 |
| Control myotubes | 5.2 ± 1.2 | 0.4 ± 0.1 | 75 ± 3.0 |
| IGF-I myotubes | 16.4 ± 2.0* | 2.0 ± 0.25* | 179 ± 8.0* |

compared with control myotubes. These findings are consistent with an increase in glycolytic metabolism in hypertrophied myotubes. To confirm this metabolic switch, the end-product of anaerobic glycolysis, lactate, was measured. In IGF-I-transfected myotubes, there was an increase in lactate levels ($179 \pm 8.0$ versus $75 \pm 3.0$ mg/l; $P < 0.001$) compared with control myotubes (Table 3). No difference was seen in lactate levels in control and IGF-I-transfected myoblasts.

### MNF expression is unaltered during myotube hypertrophy

$\alpha$- and $\beta$-MNF expression levels are summarized in Figure 5. Significant levels of $\alpha$-MNF expression were observed in both control and IGF-I-transfected myoblasts and myotubes. In contrast, $\beta$-MNF expression was not detectable during the myoblast stage in control or IGF-I-transfected cells. Post-differentiation, low levels of $\beta$-MNF expression were observed in myotubes, although the level of expression did not differ between control and IGF-I-transfected cells. These results indicate that MNFs are not directly involved in the hypertrophy process.

## DISCUSSION

Skeletal-muscle hypertrophy is an important process that occurs in both physiological and pathological states. Whereas significant advances have been made in the understanding of the molecular and biochemical processes underlying normal myogenesis [31,32], relatively little is known about these processes in hypertrophy. An important reason for this has been the absence of a quantitative cell-culture model of skeletal-muscle hypertrophy. The present study evaluates the ability of IGF-I stably transfected into C2C12 cells to produce myotube hypertrophy.

The insulin-like growth factors (IGF-I and IGF-II) are produced by many tissues and are essential in both embryonic and post-natal development [33–35]. In the mouse, the growth-promoting function of IGF-II is restricted to embryogenesis [34]. In contrast, IGF-I mediates many of the effects of growth hormone both peri- and post-natally, being involved in both mitogenesis and myogenesis [14–16,36]. Transgenic mice over-expressing IGF-I have enhanced body growth with an increase in muscle mass [37]. In contrast, mice deficient in IGF-I are significantly smaller than their litter mates, have a severe muscular dystrophy, and most ($> 95\%$) die at birth [17]. Specifically, the IGF-I-deficient heterozygotes were 10–20% smaller in total body size and in the size of individual organs, although these organs were histologically normal. IGF-I-deficient homozygotes were dead at birth, their body weight was $< 60\%$ of their wild-type siblings, and their lungs were not inflated [17]. It is evident that in these animal models IGF-I plays a fundamental role in muscle maturation and in the development of muscle mass.

In the current *in vitro* study, IGF-I-transfected and control C2C12 cells were generated from a single cell clone. The control cells were also transfected with the same plasmid lacking only the IGF-I cDNA. Therefore, all studies compared cell lines with an identical genetic background that differed only by the presence or absence of IGF-I. Quantitative morphometric analysis showed an increase in myotube area of 67% in the IGF-I-transfected C2C12 cells compared with control cells. This increase was seen in each of the three different stably transfected IGF-I cell clones analysed. Whereas quantitative analysis has been used previously in *in vivo* models of skeletal-muscle hypertrophy [19], no previous study has utilized this method of assessment in an *in vitro* model of skeletal muscle. Hypertrophy was also seen in the normal myoblasts treated with recombinant IGF-I (250 ng/ml) added to the growth medium. A dose-related effect was evident in these studies. No hypertrophy was observed in cells treated with 25 ng/ml of IGF-I. This has some similarity to the physiological response to IGF-I seen in experimental animal models. Mice with experimental heart failure treated with IGF-I showed no induction of cardiac hypertrophy at baseline plasma levels of IGF-I (mean 229 ng/ml) but hypertrophy associated with an increase in cardiac output was seen at higher plasma IGF-I levels (mean 601 ng/ml) [38]. Importantly, normal myoblasts treated with media collected from IGF-I-secreting cells also showed a hypertrophic response, demonstrating the paracrine hypertrophic effects of IGF-I. This may be of significance in increasing the efficiency of IGF-I after direct intramuscular gene transfer *in vivo*.

Recently, the signalling pathways by which IGF-I induces mitogenesis as compared with myogenesis have been elucidated [35,39,40]. Following IGF-I binding to its receptor, at least two pathways are activated: the mitogen-activated protein kinase pathway mediates mitogenesis and the phosphatidylinositol 3-kinase pathway mediates myogenesis, and these responses can be selectively blocked with specific inhibitors of these pathways [35]. In the current study, exogenous IGF-I induced a hypertrophic response when added to myoblasts, which were then allowed to differentiate. However, when IGF-I was added at various time points following the initiation of differentiation (i.e. following the change from 20% to 2% growth medium), no hypertrophy was seen in the resulting myotubes. This lack of response of C2C12 myotubes to the exogenous addition of a growth factor has also been observed in response to other growth factors such as epidermal growth factor and basic fibroblast growth factor [41]. In contrast to these latter growth factors, where there is down-regulation of their cognate receptors post-fusion [42], IGF-I receptors are in fact up-regulated post-fusion [24,25]. Clearly there is a point that is reached either during mitogenesis or at the onset of myogenesis when the C2C12 myoblasts are no longer capable of undergoing a hypertrophic response in response to IGF-I stimulation, despite there being an up-regulation of IGF-I receptors post-fusion. This is in contrast to the *in vivo* situation seen in patients with acromegaly. In this disorder involving growth hormone/IGF-I over-production, several studies have shown that apart from developing cardiac hypertrophy, patients develop hypertrophy (in response to increased IGF-I levels) of terminally differentiated type-I and type-II skeletal-muscle fibres [43,44].

In conjunction with the morphological changes observed in this study, a quantitative biochemical marker of hypertrophy was developed based on the changes in myosin isoforms known to occur during skeletal-muscle hypertrophy. Skeletal muscle is composed of muscle fibres, of which there are two broad types, type I and type II. Type-I fibres are slow-twitch fibres that undergo aerobic metabolism and contain high concentrations of ATPase. In contrast, type-II fibres are fast-twitch fibres of which there are at least three subgroups: IIa, which are fast, oxidative fibres; IIb, which are fast, glycolytic fibres; and type IIx, which are uncharacterized. With the development of hypertrophy, the predominant isoform expressed is IIb [45]. Therefore, it was hypothesized that as IIb fibres undergo anaerobic glycolysis, there would be an increase in the activity of enzymes involved in the glycolytic pathway. The end-product of glycolysis, pyruvate, represents an important metabolic branch point, with its fate depending on the oxidative state of the cell. During relative hypoxia, e.g. skeletal muscle during exertion, pyruvate is converted to lactate via LDH. This same switch from oxidative metabolism to anaerobic glycolysis is postulated to occur during skeletal-muscle hypertrophy.

In the current study, LDH and ALT activities increased significantly in hypertrophied myotubes compared with controls. This was confirmed by demonstration of an increase in lactate levels, the end-product of anaerobic glycolysis. These assays were easily reproducible and could be utilized as simple biochemical markers of skeletal-muscle hypertrophy *in vitro*, complementing quantitative morphometric changes in myotube size.

Whereas these morphometric and biochemical changes are important in establishing a cell-culture model of skeletal-muscle hypertrophy, further characterization is required in terms of the changes in expression and regulation of genes that are specifically involved in the development of hypertrophy. Several studies have identified important gene regulators of proliferation and differentiation in normal myogenesis, e.g. members of the MyoD family of muscle-specific transcription factors (MyoD, myogenin, myf-5, MRF-4) and cell cycle inhibitors (e.g. p21) [31,32]. However, little is known about the role of such factors in hypertrophy. Addition of recombinant IGF-I to L6E9 myoblasts alters mRNA expression levels of myogenin, MRF-4, myf-5, p21 and cyclins $D_1$, $D_2$, $D_7$ [18]. Myogenin and MRF-4 transcripts decreased 1 h after the addition of IGF-I but returned to normal levels at 48 h, while markers of cell proliferation, cyclins $D_1$ and $D_2$ transcripts, increased 1 h after the addition of IGF-I but returned to normal levels at 30 h. However, these acute changes do not explain the longer-term development of hypertrophy and clearly, other muscle gene regulators are likely to be involved. One possibility may involve the re-expression of fetal/developmental genes during hypertrophy, as is seen in cardiac hypertrophy. We have studied several potential factors including the $\alpha$- and $\beta$- isoforms of myocyte nuclear factor (MNF), as well as a recently identified inhibitor of skeletal-muscle hypertrophy, myostatin (growth/differentiation factor-8) [46,47].

Skeletal muscle contains an undifferentiated stem cell pool (satellite cells) that can be mobilized to regenerate myofibres in response to physiological or pathological stress [48]. MNF is selectively expressed in both cardiac- and skeletal-muscle precursor cells during embryonic development, with expression of MNF being most abundant in satellite cells, and being involved in regulating cell proliferation and cell fate [29,48]. Specifically, $\beta$-MNF expression increases significantly after muscle injury [48]. In the current model, it was postulated that if the development of hypertrophy involved recruitment of satellite cells, then MNF expression patterns would differ between control and hypertrophied cells. $\alpha$-MNF was the predominant isoform expressed in both myoblasts and myotubes, while relatively low $\beta$-MNF expression was seen only in the myotubes, with no difference between IGF-I-transfected and control cells. This indicates that the hypertrophy observed in IGF-I-transfected myotubes is more likely to result from the enlargement of C2C12 cells during the process of differentiation from myoblasts to myotubes rather than from the recruitment of a greater number of satellite cells into myotubes, which indicates that different mechanisms are involved in muscle regeneration post-injury than in the induction of physiological hypertrophy. This conclusion is strongly supported by the finding that hypertrophy was clearly present irrespective of the number of cells (nuclei) per myotube (Table 2). We have performed similar studies to assess other potential genes involved in the process of hypertrophy *in vitro*, including those encoding $\beta$-actin and myostatin, but none have shown any significant changes in expression levels during hypertrophy (data not shown).

In summary, this study describes the development of a cell culture model of skeletal-muscle hypertrophy. This model is quantitative, based on morphometric changes in myotube size, changes in DNA and protein synthesis, and biochemical changes involving both enzymes in, and products of, anaerobic glycolysis. Further characterization of this model will allow a greater understanding of the molecular and biochemical events involved in skeletal-muscle hypertrophy.

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