

Proliferation Precedes Differentiation in IGF-I-stimulated Myogenesis

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Abstract. The insulin-like growth factors (IGFs) have dramatic and complex effects on the growth of many tissues and have been implicated in both the proliferation and differentiation of skeletal muscle cells. A detailed analysis of gene expression was performed in L6E9 myoblast cultures treated with IGF-I to dissect the early events leading to the stimulation of myogenic differentiation by this growth factor. A time course of transcript accumulation in confluent L6E9 myoblasts treated with defined media containing IGF-I revealed

an initial transient decrease in myogenic factors, accompanied by an increase in cell cycle markers and cell proliferation. This pattern was reversed at later time points, when the subsequent activation of myogenic factors resulted in a net increase in structural gene expression and larger myotubes. The data presented here support the hypothesis that IGF-I activates proliferation first, and subsequently stimulates events leading to the expression of muscle-specific genes in myogenic cell cultures.

THE activation of myogenic differentiation is the end point of a cascade of intracellular events, involving a hierarchy of regulatory genes that remove myoblasts from the cell cycle and ultimately determine the characteristic pattern of skeletal muscle gene expression. Once differentiated, adult muscle tissues retain the capacity to regenerate in response to exercise or injury by activation and proliferation of satellite cells (41, 46). This process is presumably influenced by extracellular signals, which act locally to coordinate the regeneration process. Among the potential candidates for regulation of satellite cell function during regeneration is insulin-like growth factor-I (IGF-I) (13, 37).

The insulin-like growth factors (IGFs)¹ have been implicated in many anabolic pathways in skeletal muscle, including protein synthesis, nucleic acid synthesis, and glucose uptake (for review see 20). The genes encoding IGF-I (70 amino acids) and IGF-II (67 amino acids) have been cloned and characterized (for review see 70). Both factors are clearly related to insulin at both the protein and nucleic acid levels. In the adult, IGF-I and IGF-II exert pleiotropic effects on numerous tissues, some of which mimic insulin action on cellular metabolism (for review see 43). IGF-I is distinct from insulin, however, in that it is synthesized by many tissues and has two major alternatively spliced isoforms.

Both IGF-I and IGF-II are known to also stimulate

myogenic differentiation in cell culture (14). Several muscle cell lines have been characterized that differ both in their patterns of IGF synthesis and myogenic factor expression. Although IGF-I expression is very low or nonexistent in the neonatal rat L6 line and IGF-II is synthesized only at very low levels (59), the IGF-I receptor is present, rendering this cell line useful for the study of the effects of IGF-I on muscle. L6 cells are also restricted in their expression profile of myogenic factors, and although myf-5 is present in myoblasts (5) and myogenin and MRF4 are induced upon differentiation (57, 79), MyoD is not expressed (5, 57). In contrast, the adult mouse C2C12 line expresses MyoD and myogenin (9, 11, 57), as well as both IGF-I and IGF-II (71, 72). This could explain why differentiation of the C2C12 cell line is less responsive to the application of exogenous IGFs (21).

A relationship between IGF and myogenic factor expression was established by Florini and colleagues (17). Their study showed that when rat L6 muscle cells were exposed to myogenin antisense oligomers, the cells became refractory to stimulation of differentiation by IGF-I, although other anabolic stimulatory effects of the growth factor were unaffected. Differentiation, as measured by muscle creatine kinase levels, was proportional to the level of myogenin gene expression induced in these IGF-I-treated cells (21). In a complementary study, the introduction of either IGF-I or IGF-II antisense oligonucleotides into C2C12 cells partially blocked differentiation, suggesting that both IGFs may play a critical role in myogenic differentiation (22).

IGF-I is defined as a growth factor because of its mitogenic capability. Most cell lines characterized to date require IGF-I for cell division (for review see 43). Specifically, IGF-I is required for cells to traverse the G1 phase of the cell cycle, and it is therefore referred to as a progres-

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1. *Abbreviations used in this paper:* cdk, cyclin-dependent kinase; DAPI, 4',6-diamidino-2-phenylindole; β -gal, β -galactosidase; HS, horse serum; IGF, insulin-like growth factor; MHC, myosin heavy chain; MLC, myosin light chain.

sion factor. However, IGF-I cannot act alone and requires the presence of, but is distinguished from, competence factors that permit a cell to enter the G1 phase from the G0 or quiescent phase (51). In addition to a progression factor, protein synthesis is also required to initiate the S phase of the cell cycle. If protein synthesis is blocked, the treatment of cells with IGF-I fails to elicit a procession through the "V checkpoint" of the G1 phase of the cell cycle (51).

The role of the cell cycle in myogenesis has recently been the subject of intensive study (for review see 40). Terminally differentiated skeletal muscle cells are thought to be irreversibly withdrawn from the cell cycle (49); i.e., fused, multinucleated myotubes no longer synthesize DNA or divide. Regulation of the cell cycle is governed, in part, by Rb, the retinoblastoma gene product. In particular, differentiated myotubes lacking Rb will continue to synthesize DNA (64). Additional regulation of the cell cycle is controlled by the cyclins, a class of proteins believed to promote progression through the cell cycle by activating cyclin-dependent kinases (cdks). Among these, the D-type cyclins (D1, D2, and D3) have emerged as key regulators of the G1 phase of the cell cycle in eukaryotes (for review see 65). They presumably share overlapping but not completely redundant activities, as evidenced by their tissue-specific distribution (for review see 65). The ectopic expression of one of these cyclins, cyclin D1, has also been shown to inhibit myogenic factor activation of muscle-specific reporter constructs (56, 67). Conversely, the cell cycle inhibitor, p21, which is also known to bind to the cdks (27, 32, 80), can be activated by MyoD, and thus may play a role in linking myogenesis with cell cycle withdrawal (29, 52, 67).

It has been previously demonstrated that IGF-I stimulates both proliferation and myogenesis in the L6E9 cell line. The present study was undertaken in an attempt to resolve this paradoxical effect of IGF-I on myoblasts in tissue culture. To characterize the cell cycle events associated with IGF-I stimulation of confluent L6E9 cells, we assayed the mRNA expression pattern of nuclear factors during myogenesis. The transcript levels of D-type cyclins were initially stimulated by IGF-I, but at later time points these levels were diminished until they were equivalent to those of untreated cells. This mRNA expression pattern was the inverse of that for the myogenic factors, myogenin and MRF-4, which were initially repressed by IGF-I, but later were equivalent to the expression from untreated cells. The application of exogenous IGF-I to myoblasts resulted in larger myotubes and the activation of a myosin light chain reporter construct (MLC1CAT). At 48 h the IGF-I-treated cells expressed myogenin equivalent to the levels observed for untreated cells, despite having a proliferative response as great as cells maintained in growth media (20% FCS). These data indicate that although IGF-I-treated myoblasts remain in the proliferative state longer, and therefore initiate fusion later, they exhibit an accelerated and enhanced myogenic response.

Materials and Methods

Cell Lines and Culture Conditions

The L6E9 cell line, a subclone of the rat L6 neonatal cell line (81), was se-

lected for its ability to fuse quickly into terminally differentiated myotubes (49). Cells were maintained as myoblasts by culturing in DME with 20% FCS. Penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD) was included in the media at 50 U/ml and 50 µg/ml. Cells were differentiated in serum-free DME, or in DME supplemented with 2% horse serum (HS), or DME supplemented with the indicated concentrations of IGF-I. IGF-I (Ciba-Geigy, Research Triangle Park, NC) was generously provided by J. Florini (Syracuse University, Syracuse, NY). 500 µg/ml BSA was added with the IGF-I as described by Florini and Ewton (17).

Immunohistology

L6E9 cells were maintained and treated with IGF-I under the conditions described above. The cells were lysed with PBS/0.1% Triton X-100 and fixed in 100% methanol. After blocking with horse serum, cells were incubated with anti-myosin heavy chain (MHC) mAb, MF-20 (gift from D. Bader, Vanderbilt University, Nashville, TN), overnight at 4°C. Cells were then washed with PBS and treated with biotinylated secondary antibody, avidin, and biotinylated HRP according to the manufacturer's instructions (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA). Cells were photographed with a phase-contrast microscope (Nikon Inc., Garden City, NY).

Expression Vectors, Transfections, and CAT Assays

MLC1 promoter-CAT expression vectors carrying the 173-bp myosin light chain (MLC) enhancer (pCAT173) were described previously (76). Transfections of CAT reporter constructs and pSV-β-galactosidase normalizing vector (catalogue E1081; Promega, Madison, WI) were performed by calcium phosphate coprecipitation as described previously (60). 100-mm plates were transfected with 15 µg of pCAT173 construct, the indicated amount of myogenic factor expression vector, pSV-β-galactosidase, and vector DNA for a total of 30 µg DNA per plate. Cells were switched to differentiation media 16 h after DNA/calcium phosphate precipitate had been added and were harvested after an additional 48 h. CAT assays were performed using normalized amounts of cell extracts. Total protein was measured spectrophotometrically by reacting 1 µl of cell extract with protein assay dye reagent (catalogue 500-0006; Bio Rad Laboratories, Hercules, CA). β-galactosidase (β-gal) assays were performed on extract volumes normalized to the total protein concentration of each extract. The β-gal activities were then used to determine the volume of cell extract used in subsequent CAT assays. CAT assays were performed according to published protocols (60).

Northern Analysis

Total cellular RNA was isolated from L6E9 cells grown on tissue-culture plates using guanidinium lysis and CsCl gradients. 20 µg of RNA was loaded in each lane of a 1.5% agarose gel containing 5% formaldehyde and electrophoresed in a 1× MOPS (20 mM) buffer. RNA was transferred to Zetabind nylon membrane (Cuno, Inc., Meriden, CT). Prehybridization and hybridization were performed overnight at 42°C in 50% formamide according to the manufacturer's instructions. Probes were prepared from full-length cDNA fragments using a multiprime DNA labeling kit (Amersham Corp., Arlington Heights, IL) to incorporate [α -³²P]dCTP (3,000–6,000 Ci/mmol) (New England Nuclear, Boston, MA). Ethidium bromide staining was used to verify equal loading. Quantitation of the Northern blots was done using NIH Image software.

DNA Synthesis Assays

DNA synthesis was measured by [³H]thymidine uptake done according to published protocols (18). Briefly, 25 µl of [³H]thymidine (6.7 Ci/mM) (catalogue NET-027; Dupont-New England Nuclear, Wilmington, DE) was added to each plate of tissue-culture cells, and cells were replaced in the incubator. To harvest, cells were washed twice with PBS, and then 10% TCA was added to each plate for 5 min. The plates were then washed twice with water, and 200 µl of .25 M NaOH was added. Plates were then incubated for 1–2 h, scraped, and measured in a scintillation counter.

4',6-Diamidino-2-phenylindole (DAPI) Nuclear Staining

DAPI (catalogue 236 276) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used according to the manufacturer's

directions. Briefly, cells were washed two to three times in PBS, fixed in ice-cold methanol for 5 min, and then washed in PBS two to three times to rehydrate. The plates were incubated in 1 $\mu\text{g/ml}$ of DAPI for 15 min, and then rinsed with PBS. Nuclear staining was visualized on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) with a DAPI filter. Eight separate randomly selected fields were counted to quantitate nuclei in each experimental group.

Results

IGF-I Enhances Myogenesis in L6E9 Myoblasts

In the present study, a subclone of the original L6 line (L6E9) was used to study the molecular basis of IGF-I-accelerated myogenesis. The L6E9 subline was originally selected for its ability to fuse quickly, and the contractile protein isoforms it expresses have been characterized (26, 48, 50). To examine the effects of IGF-I on the myogenic properties of the L6E9 line, we differentiated confluent L6E9 myoblast cultures in defined media containing increasing amounts of IGF-I (see Materials and Methods). Clear differences in cell morphology were observed with the varying levels of IGF-I treatment. Cells in defined media that contained 25 ng/ml or higher concentrations of IGF-I had larger multinucleated myotubes than those seen in control plates (Fig. 1). These morphological indicators of myogenesis were reduced at the highest IGF-I concentrations (150 ng; data not shown), but they were still greater than in conditions lacking IGF-I. The biphasic response of myoblasts to IGF-I is in agreement with previous studies in which enhanced differentiation by IGF-I was demonstrated by increased muscle creatine kinase gene expression in L6A1 cultures (19). Having established a similar response to IGF-I in the L6E9 subline, we used these cells for the subsequent experiments described below. In these studies, we focus on the response of confluent L6E9 myoblasts to IGF-I.

A Transfected MLC Enhancer Is Activated in L6E9 Myotubes by Exogenous IGF-I

To investigate the molecular pathways involved in IGF-I-enhanced myogenesis, we examined the effect of exogenous IGF-I on the activation of a muscle-specific enhancer associated with the rat MLC 1/3 locus. This enhancer lies downstream of the genes encoding the MLC1 and MLC3 isoforms (10, 63), which are transcribed from two differentially regulated promoters with accompanying alternate splicing pathways (53, 69). The essential role of the enhancer in activating MLC transcription has been demonstrated in muscle cell cultures (10, 63, 76). In addition, transgenic mice carrying a CAT reporter linked to the MLC1 promoter and the downstream MLC enhancer express the CAT transgene exclusively in skeletal muscle tissues (62). Thus the MLC enhancer constitutes a marker for myogenesis and therefore a potential molecular target for the action of IGF-I. Since L6E9 cells do not normally express MLC1 (26, 50), they represent an ideal model for examining the possible activation of the MLC enhancer by exogenous stimuli.

A rat MLC1 promoter-CAT reporter construct, driven by a rat MLC enhancer (pCAT173) (76), was cotransfected with a pSV-*lacZ* normalizing vector into L6E9 myo-

blasts. The transfected cells were differentiated in defined media containing varying concentrations of IGF-I. Since IGF-I causes a general increase in transcription and metabolism (1, 15, 18), β -gal assays were normalized to protein levels in the extracts, and then the amount of extract used in each CAT assay was normalized to these β -gal values. The action of IGF-I on the transfected CAT gene construct could therefore be distinguished from a general stimulation of metabolic rates. As seen in Fig. 2, IGF-I induced expression from the pCAT173 construct in a titratable fashion. When treated with 100–150 $\mu\text{g/ml}$ of IGF-I, the activity from pCAT173 demonstrated an approximately fourfold induction over the activity from pCAT173 in untreated cells.

The MLC Enhancer Is Activated in L6E9 Myotubes by Forced Myogenin Expression

Previous studies aimed at defining the role of myogenin in IGF-I-enhanced myogenesis have shown that exposure of L6A1 cells to exogenous IGF-I is accompanied by an increase in myogenin mRNA (21), and that stimulation of myogenesis in these cells is specifically blocked by an antisense oligonucleotide complementary to the myogenin coding region (17). These results support the hypothesis that IGF-I-enhanced myogenesis operates through a pathway that involves the action of myogenin. Notably, the L6E9 cell line used in this study expresses myogenin but not MyoD (5, 79). The inability of this cell line to activate the MLC enhancer during differentiation could therefore be due to the absence of MyoD. Thus, it can be envisioned that levels of active, endogenous myogenin in L6E9 cells may be below a threshold necessary to induce MLC enhancer activity. We therefore investigated whether an increase in intracellular myogenin concentration could activate the MLC enhancer in L6E9 cells. The pCAT173 construct described above was cotransfected with increasing amounts of either MyoD or myogenin expression vectors into L6E9 cultures. As seen in Fig. 3, cotransfection of these cells with either a MyoD or a myogenin expression vector together with the MLC1 enhancer-CAT construct induced CAT activity. Increasing the amounts of both myogenin and MyoD expression plasmid led to increased activity from the MLC reporter construct. This further suggested that myogenin may be involved in the IGF-I-induced activation of the MLC enhancer. MyoD is a less likely candidate because it is not expressed normally in L6E9 cells (5, 79).

IGF-I Treatment Initially Represses Myogenin and MRF4 Expression in L6E9 Cells

To establish a relationship between the action of IGF-I and the induction of MLC enhancer activity by myogenic factors, we investigated whether IGF-I increases accumulation of myogenic factor transcripts in L6E9 cultures. Total RNA was prepared from L6E9 cells harvested at different times after IGF-I treatment. Northern blot analysis in Fig. 4 A demonstrated an approximately threefold decrease in myogenin transcripts after 1 h of IGF-I treatment (*second and third lanes from the left*), and it remained slightly repressed through 30 h of treatment. By 48 h, myogenin transcript levels were equivalent in the IGF-I-

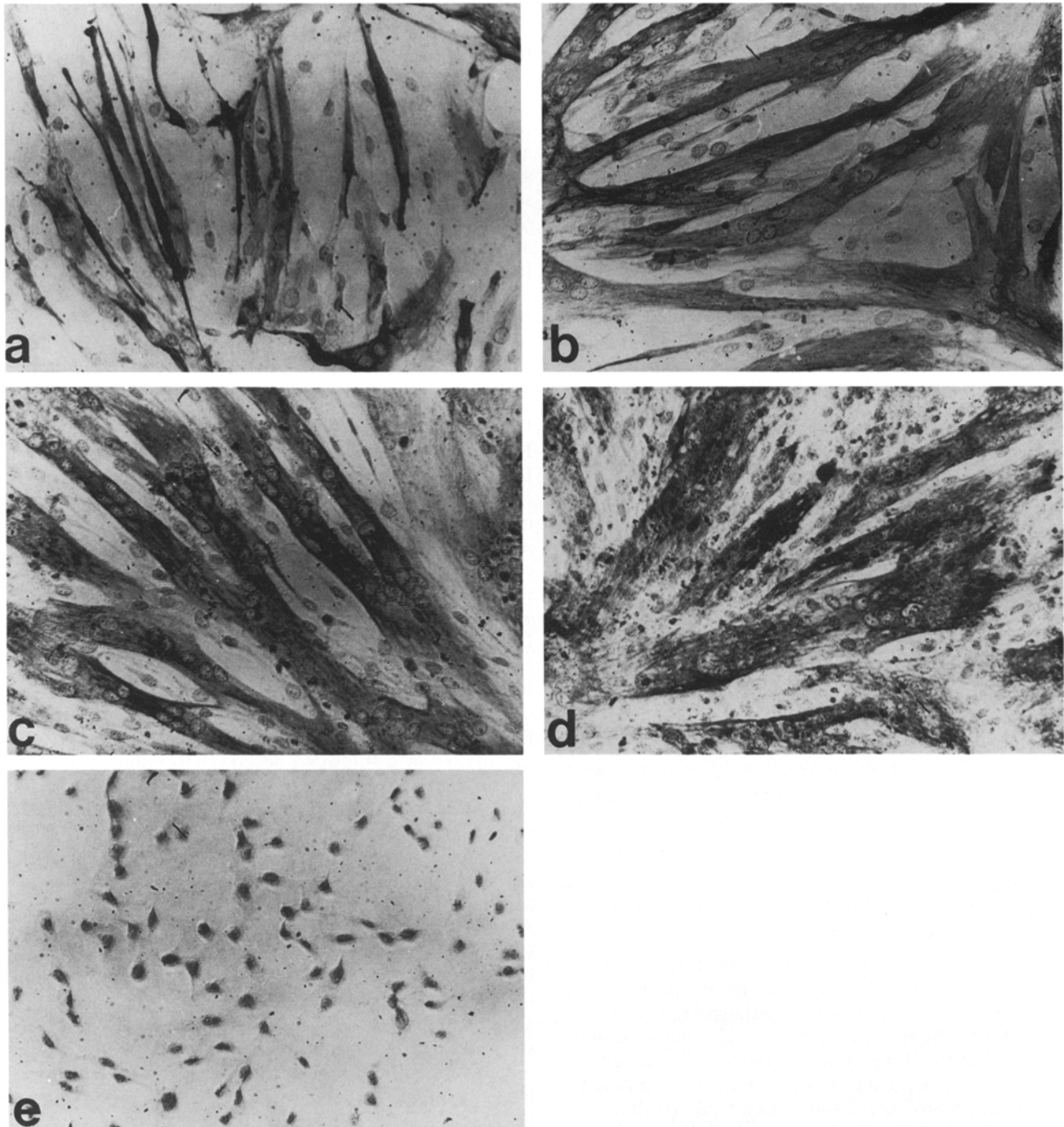


Figure 1. IGF-I enhances myogenic differentiation of L6E9 cells. Parallel cultures of L6E9 myoblasts were treated with different concentrations of IGF-I in defined media (DME). After 48 h, the cultures were fixed and stained with an antibody against MHC (MF-20) as described in Materials and Methods. (a) Cells were differentiated in the absence of IGF-I. Cultures shown in b, c, and d were treated with 25, 50, and 100 ng/ml IGF-I, respectively. Cultures shown in e are myoblasts maintained in growth medium.

treated and the untreated cultures. A similar decline in MRF4 transcripts was observed (Fig. 4 A). MRF-4 transcript levels, however, were still substantially repressed at 30 h, but by 48 h, they had also become equivalent to that of untreated cells. Although already at low levels, the expression of myf-5 mRNA was diminished (Fig. 4 A) as previously reported (44), and it was not affected by IGF-I treatment. These results are inconsistent with a direct in-

duction of myogenic markers by IGF-I and indicate that myoblasts have a multiphasic response to the growth factor.

IGF Activates Cyclins D1 and D2

To determine if markers of cell proliferation were affected by IGF-I treatment of L6E9 myoblasts, we investigated the level of the mRNA of the D-type cyclins at early time

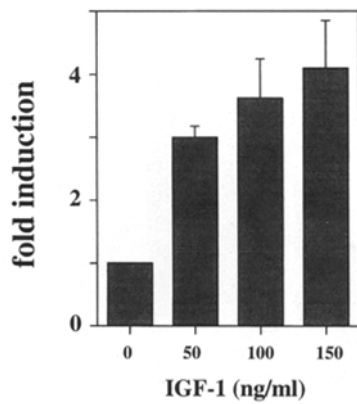


Figure 2. IGF-I induces MLC enhancer-driven CAT activity in L6E9 cells. L6E9 myoblasts transfected with MLC-CAT construct pCAT173 were treated with increasing concentrations of IGF-I as indicated. Cultures were harvested at 48 h, and cell extracts used for CAT assays were normalized to β -gal activity adjusted for total protein (as measured by Bradford assay). Results are expressed as fold induction relative to untreated cultures. Values are the average of at least three experiments (with standard error).

points. It has previously been demonstrated that the D-type cyclins respond to growth factors (25, 45, 77, 78). In addition, cyclin D1 has recently been implicated in the repression of myogenic factor transactivation of muscle-specific genes in 10T1/2 cells (56, 67). As seen in Fig. 3, transcripts encoding cyclins D1 and D2 increased by 1 h after treatment with IGF-I, and a substantial increase was maintained through 8 h after treatment. After 30 h of treatment, the level of cyclin D1 and D2 expression was equivalent to that of the untreated cells. Transcripts encoding cyclin D3, which has been previously shown to increase during myogenesis in L6 cells (39), were not affected by IGF-I during this time period. An additional cell cycle regulator, p21, inhibits cell cycle progression by binding to cdk complexes

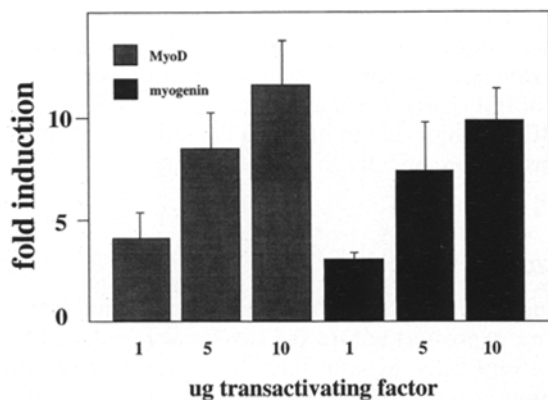


Figure 3. Cotransfection of either MyoD or myogenin transactivates the MLC enhancer in L6E9 cells. L6E9 myoblasts were cotransfected with pCAT173 and either a MyoD (shaded bars) or myogenin (black bars) expression vector. Results are expressed as fold induction relative to cultures transfected with vector DNA alone. Each value (with standard error) represents the average of at least two experiments.

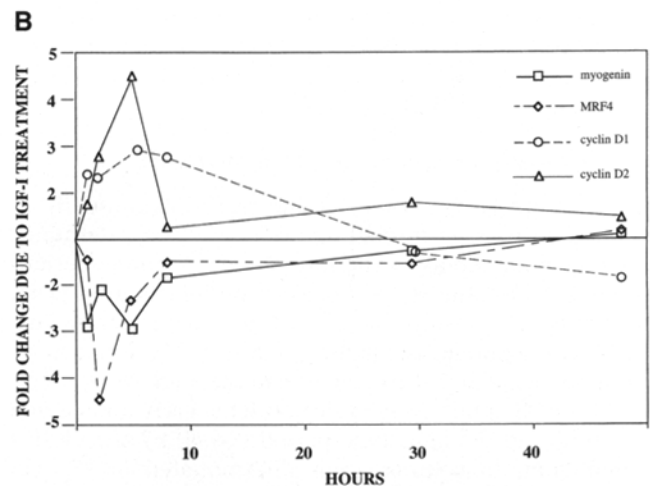
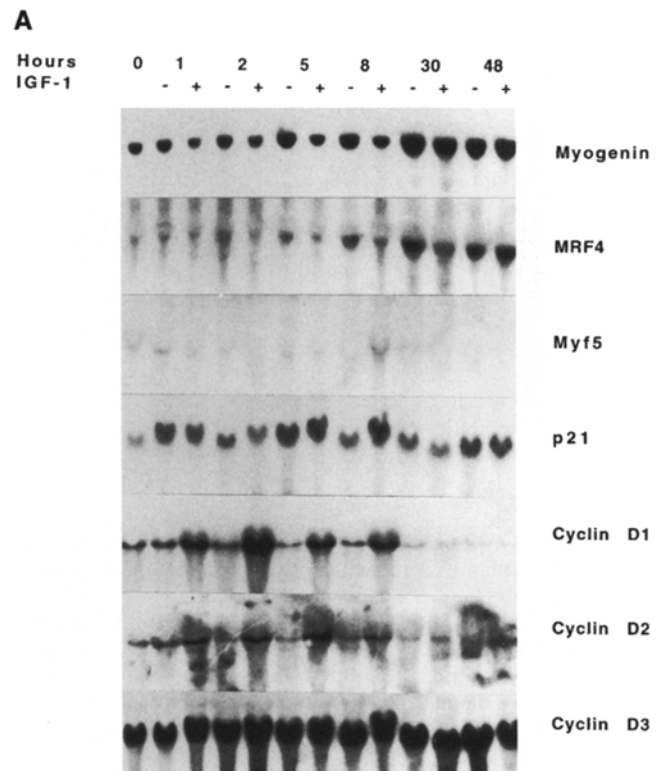


Figure 4. IGF-I modulates accumulation of transcripts encoding markers of myoblast proliferation and differentiation. (A) Total RNA, extracted from L6E9 cells, either untreated (-), or treated with IGF-I (+), was analyzed by Northern blots with the indicated 32 P-labeled probes. The leftmost lane shows RNA from confluent L6E9 cells maintained in 20% FCS (0). At this time point, other plates were shifted to DME alone (-) or DME with IGF-I (+) and harvested after the indicated times. The low levels of myogenin and MRF-4 seen in the lane representing the 0 time point are a result of the confluency of the L6E9 cultures. (B) The results for four of the transcripts shown in Fig. 4 A (myogenin, MRF-4, cyclin D1, and cyclin D2) were quantified by densitometric scan. The level of mRNA was plotted from IGF-I-treated L6E9 cells relative to the level from untreated cells at each time point.

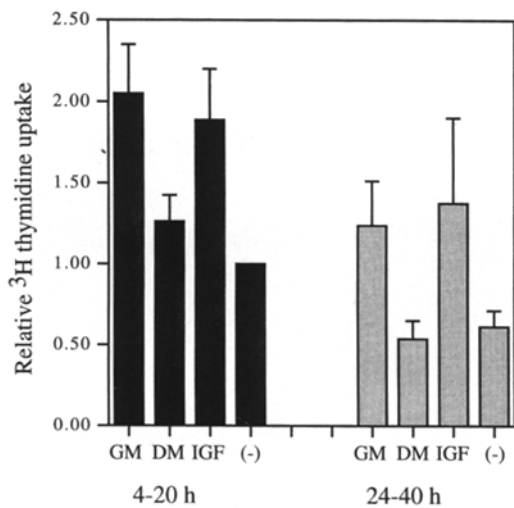


Figure 5. IGF-I stimulates DNA synthesis in the absence of serum. L6E9 myoblasts were incubated for 16 h with [³H]thymidine in either 20% FCS (GM), 2% HS (DM), IGF-I (IGF), or DME alone (-). Incorporated [³H]thymidine was measured over two time frames, 4–20 h (black bars) and 24–40 h (shaded bars). Results are expressed relative to the cultures in DME alone during the 4–20-h time frame. Values are the mean of three experiments (with standard error).

(27, 32, 80), and its induction has recently been correlated with myogenesis (28, 29, 39, 52). In our experiments, however, no change was seen in p21 expression in the 48 h after treatment with IGF-I (Fig. 4 A).

IGF-I Treatment Stimulates DNA Synthesis

Since an increase in D-type cyclin transcripts is usually indicative of passage through the cell cycle, we determined [³H]thymidine uptake experiments over two time periods after IGF-I treatment, as a measure of the extent of DNA synthesis. The results shown in Fig. 5 demonstrate that IGF-I was a potent stimulator of DNA synthesis in confluent L6E9 cells. IGF-I treatment had the equivalent effects of 20% FCS in this assay during both the early time period (4–20 h) and the later time period (24–40 h) after IGF-I stimulation. In contrast, cells differentiated in 2% HS demonstrated no increase in [³H]thymidine uptake over basal levels. Thus, IGF-I stimulates DNA synthesis in L6E9 myoblasts even when applied to confluent cells.

IGF-I Treatment Initially Promotes Cell Division of L6E9 Myoblasts

To assess the cumulative effects of the increased DNA synthesis observed in Fig. 5, we measured the increase in nuclei caused by IGF-I treatment. Confluent L6E9 myoblasts were stained with DAPI at 24 h and 48 h after IGF-I treatment. These experiments indicated that the increased DNA synthesis observed in IGF-I-treated cells correlates with an increased proliferation after 24 h (Fig. 6). At 24 h, the number of nuclei in IGF-I-treated cultures was significantly greater than cells cultured in either 2% HS supplemented media or in DME alone but only slightly more than the number in 20% FCS-treated cultures. By 24 h, some fusion and myotube formation could be seen in the

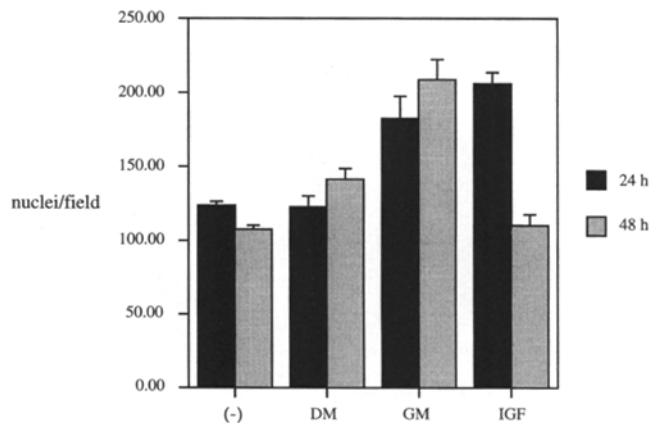


Figure 6. IGF-I stimulates L6E9 cells to divide, but not all the cells survive. L6E9 myoblasts, passaged in 20% FCS, were transferred to either 20% FCS (GM), 2% HS (DM), IGF-I (IGF), or in DME alone (-). Cell cultures were stained with DAPI at either 24 h (black bars) or 48 h (shaded bars). The nuclei in eight randomly selected fields were counted in each experiment. Values are the mean of three experiments (with standard error).

DME and 2% HS cultures plates, but no fusion was observed in the 20% FCS or IGF-I-treated plates (data not shown). After 48 h, the IGF-I-treated cells had ~50% fewer nuclei than after 24 h of treatment. The 20% FCS, 2% HS, and DME cultures did not exhibit this extensive reduction in nuclei (Fig. 6). Thus, the enhanced myogenesis demonstrated in Fig. 1 was accompanied by a significant reduction in the total number of nuclei that was not seen in the other treatment groups.

The IGF-I stimulation of myogenesis is not due to mitogen depletion. To assess the possibility that the enhanced myogenesis induced by IGF-I was the indirect result of proliferating cells gradually depleting the media of IGF-I, experiments were performed in which IGF-I was reapplied every 12 h to the differentiating L6E9 cells. The myogenesis that resulted from the addition of IGF-I to cells only once was compared to cells to which fresh IGF-I-containing media was added every 12 h. As shown in Fig. 7, the further addition of fresh IGF-I stimulated an even greater myogenic response at 48 h (C) than the single application of IGF-I (B). This indicates that the myogenic activity of IGF-I is not due to mitogen depletion, but rather that it participates directly in regulating myogenic differentiation.

Discussion

In this study, we have identified specific mitogenic markers that are expressed before the myogenesis induced by IGF-I in L6E9 cells. Several lines of evidence implicate proliferation as a precursor to myogenesis during IGF-I-stimulated differentiation. First, cyclin D1 and cyclin D2 were activated within the first hour of IGF-I treatment. Second, IGF-I caused the cells to go through the S phase of the cell cycle as demonstrated by tritiated thymidine uptake experiments. Third, as demonstrated by DAPI staining, the IGF-I-treated cells completed another round of the cell cycle and possessed more nuclei than untreated

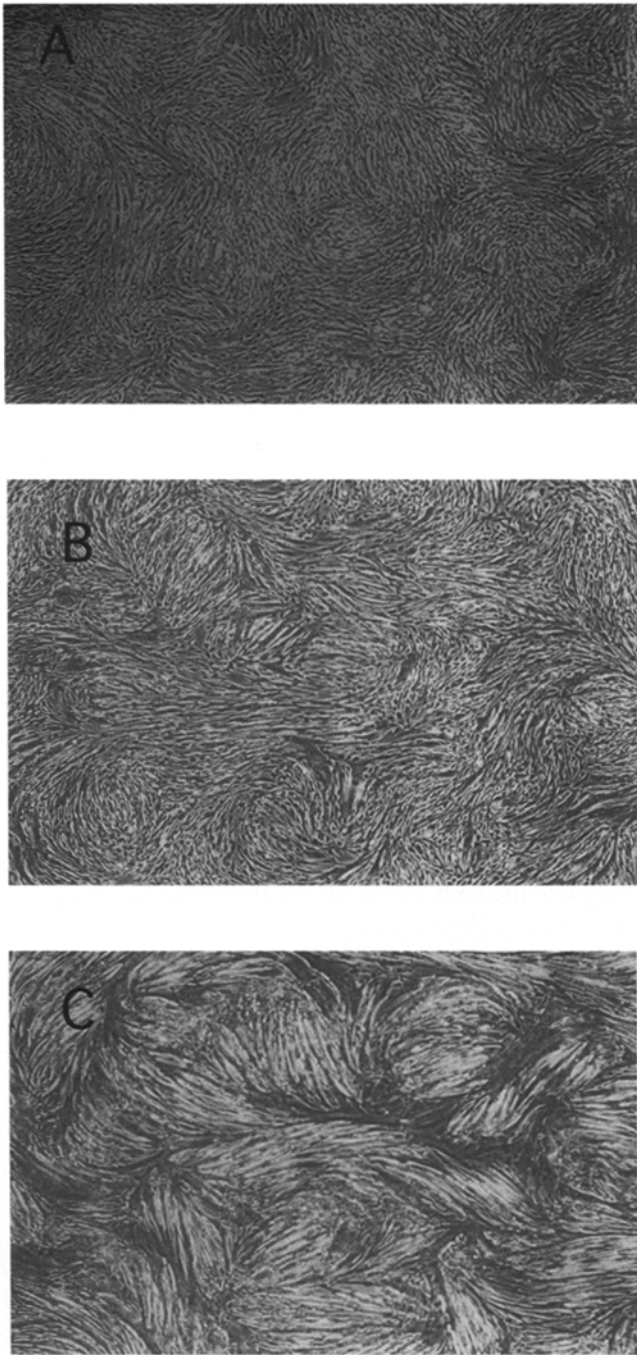


Figure 7. Myogenic stimulation by IGF-I is not dependent on IGF-I depletion. Confluent L6E9 myoblasts were switched to either DME alone (A) or DME supplemented with 100 ng/ml IGF-I (B and C). One experimental group was treated with additional IGF-I every 12 h (C). After 48 h, the cells were fixed and photographed.

cells at 24 h after treatment. Fourth, the transcriptional activation of the myogenin and MRF4 genes was delayed in IGF-I-treated L6E9 cells. Although the levels of myogenin and MRF-4 are initially suppressed by IGF-I treatment, the levels after 48 h of treatment are equivalent to those of untreated cells, indicating that this suppression is followed by an accelerated progression of myogenesis.

The activation of myogenic factor expression appeared to be inversely correlated with the expression of cyclin D1 and D2 (Fig. 4 B). Thus, the balance of nuclear factors, both proliferative and myogenic, may play a critical role in the final steps of the signaling pathway initiated by IGF-I. Taken together with previous findings, the present study indicates that the myogenesis stimulated by IGF-I occurs later than, but is perhaps dependent on, the earlier mitogenic stimulation.

In contrast with myoblasts cultured in DME alone, myoblasts treated with IGF-I resulted in the formation of larger myotubes, despite their later exit from the cell cycle. IGF-I, like insulin, causes an increase in protein synthesis and a concurrent decrease in protein degradation in myoblasts (15, 58). Here we demonstrate that treatment of L6E9 myoblasts with increasing amounts of IGF-I results in increased cellular hypertrophy (Fig. 1). This effect is not limited to skeletal muscle cells, as IGF-I expression correlates with hypertrophy in smooth muscle (6) and induces hypertrophy in cardiomyocytes (34). The IGF-I-stimulated hypertrophy of L6E9 myotubes is consistent with earlier work that demonstrated that primary avian myofibers undergo hypertrophy when stimulated with IGF-I (74). In addition, Schwartz and colleagues have shown that muscle-specific expression of an IGF-I transgene results in hypertrophied muscles (7).

It has been demonstrated that DNA synthesis and cell division are not prerequisites for the myogenic effect of IGF-I (73). In fact, recent work suggests that the mitogenic effect of IGF-I actually may delay myogenesis. Florini and colleagues recently demonstrated that myogenic cells plated at a higher density have a greater myogenic response to IGF-I than cells plated at a lower density (16). These authors hypothesize that the increased response to IGF-I by cells plated at high density was actually due to the inhibition of mitogenesis, allowing for earlier myogenesis. However, the increase in cell density caused by the IGF-I-stimulated mitogenesis could also contribute to an increase in myogenic cell-cell contacts, thereby enhancing the IGF-I stimulation of myogenesis seen in this study and in the present one.

Quinn and Roh have also demonstrated that in L6 cells overexpressing the IGF-I receptor, high concentrations of IGF-I led to an increased mitogenic response and, in fact, prevented the cells from forming myotubes (54). This is in agreement with other studies demonstrating that at high doses of IGF-I, when L6 cells exhibit maximum proliferation, the stimulated differentiation was less than at low concentrations (19). The number of IGF-I receptors has been shown to increase in myoblasts during differentiation (71). Thus, it is possible that the mitogenic and myogenic activities of IGF-I are mediated by both the IGF-I concentration and the IGF-I receptor density.

In this work, we demonstrated that the high doses of IGF-I, even when reapplied to cells, resulted in an enhanced myogenesis (Fig. 7). This is in agreement with previous work that demonstrated an even greater stimulation of myogenin, and no increase in Rb phosphorylation when IGF-I is reapplied after 24 h (61). Thus, the myogenic effect of IGF-I was not due to the proliferating cells depleting the media of IGF-I.

A candidate for mediating the myogenic induction by

IGF-I is myogenin. The enhanced MLC reporter activity in IGF-I-treated L6E9 cells, where the MLC locus is normally inactive, is a further indication of an increased myogenic response that could depend on myogenin. We have previously demonstrated that exogenously expressed myogenin can activate the MLC enhancer in NIH3T3 cells (76), and in this study we show that myogenin can activate it in L6E9 cells (Fig. 3). In addition, earlier work implicated myogenin in the induction of differentiation by IGF-I (17, 21). In the present study, however, the expression of myogenin in IGF-I-treated L6E9 cells was actually repressed as early as 1 h after treatment, and at 48 h was only equivalent to the expression from untreated cells. Only at later time points did the level of myogenin mRNA in IGF-I-treated cells ever exceed the level in untreated cells (data not shown). These data are consistent with the initial repression and later recovery of myogenin expression seen by Rosenthal and Cheng (61). Although myogenin mRNA increased in untreated L6E9 cells, this was not accompanied by the larger myotubes and increased muscle-specific gene activity characterizing L6E9 cells treated with IGF-I (23) (Figs. 1 and 2). Taken together, these results demonstrate that myogenin expression is necessary but clearly not sufficient for the enhanced myogenesis caused by IGF-I.

Myoblasts may require the continuous repression of myogenic factors below a threshold level to complete the cell cycle. Cyclin D1 has been shown to repress the activity of MyoD (56, 67) and of myogenin (56) in 10T1/2 cells. The early repression of myogenin and MRF-4 transcripts observed in this study coincides with the accumulation of cyclin D1 and D2 mRNA. In this report, we have demonstrated that the mRNA levels of cyclin D1 and cyclin D2 are induced within the first hour of treatment by IGF-I, with the peak of expression occurring between 2 and 5 h after treatment. Rosenthal and Cheng have also found that IGF-I also stimulated the expression of cyclin D1 mRNA in L6 cells (61). Consistent with these results, it has been shown that in other cell lines, cyclin D1 and D2 respond to mitogenic signals (45, 77, 78), including IGF-I (25). Other studies (35, 55, 75) have demonstrated that expression of cyclin D1 mRNA is reduced to very low or undetectable levels at later stages of myogenesis. The initial stimulus by IGF-I is similar to the response of myoblasts to FGF in which cyclin D1 also increased significantly by 4 h after treatment (55). However, in contrast with the effects of IGF-I, the expression of myogenic factors remains repressed in FGF-treated cells, and these cells do not go on to become myotubes and do not express the muscle-specific marker MHC (55). Thus, a balance between growth and differentiation in myoblasts could be mediated by the sequential activation of nuclear factors responding specifically to IGF-I.

We detected no consistent change in the expression level of p21 mRNA during the 48 h of IGF-I treatment (Fig. 3). Recently, a correlation has been observed between the expression of myogenic factors and cdk inhibitors, such as p21, that prevent passage through the cell cycle. The expression of p21 was shown to increase during myogenesis in C₂C₁₂ cells (28, 29, 52) and can be activated by the myogenic factor MyoD in CV1 cells (29). However, in a similar study, Hamel and colleagues showed that p21

mRNA is not induced in L6 cells until after 48 h in differentiation media (39). Thus, while p21 may still play a role in the terminal differentiation of L6E9 cells, it is likely that its effect takes place after the myogenic program is well established.

The steep decline in the total number of nuclei in the IGF-I-treated plates after 48 h was an unexpected result. It is not clear what contributes to this decline, but it is possible that only the cells that divided survived. This idea is supported by the fact that the tritiated thymidine uptake in the IGF-I-treated plates was equivalent to the plates cultured in 20% FCS even though these plates demonstrated no cell death or myogenesis. Although apoptosis has recently been implicated in myogenesis (47), most reports support IGF-I's role in preventing apoptosis (2, 33). Additionally, IGF-II has been shown to inhibit programmed cell death in the muscles of mdx mice (68). Whatever the source of the cell death seen in this study, it does not preclude the robust myogenesis seen in IGF-I-treated L6E9 cells. It remains a formal possibility that IGF-I treatment could select for a more myogenic subset of the parental line; however, the initial proliferative response to IGF-I, equivalent to the levels of proliferation induced by serum, argues against this interpretation.

The results presented in this study have potentially important implications for the developmental induction of the myogenic phenotype. During mammalian embryogenesis, IGF-I expression is very low in most tissues, although IGF-I receptor mRNA is present in muscle (4). In particular, myogenic cells are only weakly positive for IGF-I mRNA in the embryo (3, 30, 31) and in the neonate (37). Nevertheless, Han et al. (31) found the IGF-I peptide localized to embryonic myoblasts, even though there was no detectable mRNA expression. They concluded that even at these early stages, IGF-I could be released from neighboring cells and absorbed by IGF-I receptors on myoblasts. This conclusion was supported by their finding that connective tissue surrounding skeletal muscles was found to be positive for IGF-I mRNA (12, 30, 31). The important role of the IGFs in development was further highlighted by the phenotype of mice lacking the IGF-I receptor. These mice failed to develop to their full size, remaining ~60% of the size of normal mice from birth onward (42). In particular, a reduction in the number of myocytes was observed in this study. Interestingly, targeted mutation of the cyclin D1 gene also displayed a phenotype of reduced growth, among other defects (66). The developmental coordination of cell division and myogenesis may depend on the response of myogenic cells to the timed release of IGF-I.

The general distribution of IGF-I expression suggests that it may play an important role in tissue-specific metabolic functions, not only in development but also in the adult mammal through autocrine and/or paracrine pathways (for review see 8). One such paracrine function of IGF-I is muscle regeneration. In adult muscle tissue, IGF-I may coordinate the synchronous differentiation of satellite cells in response to muscle injury. Increased transcription of IGF-I and accumulation of the peptide has been documented in regenerating muscle (13, 36, 38), where it presumably acts locally as a trophic factor for the recruitment of satellite cells into the differentiation process. In addi-

tion, regenerating muscle has also been shown to express increased levels of MyoD and myogenin mRNA (24). It may well be that IGF-I causes proliferation first and then differentiation: a sequence of events perfectly timed for the repair of injury by stem cells.

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