Insulin-like Growth Factor-II Is an Autocrine Survival Factor for Differentiating Myoblasts*

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Recent studies indicate that insulin-like growth factor-II (IGF-II) acts as an autocrine differentiation factor for skeletal myoblasts in culture. IGF-II mRNA and protein are induced as early events in muscle differentiation, and the rate and extent of IGF-II secretion correlate with both biochemical and morphological differentiation. Here we show that IGF-II also functions as an essential survival factor during the transition from proliferating to differentiating myoblasts. Stably transfected C2 muscle cell lines were established in which a mouse IGF-II cDNA was expressed in the antisense orientation relative to the constitutively active Moloney sarcoma virus promoter. IGF-II antisense cells proliferated normally in growth medium containing 20% serum but underwent rapid death when placed in low serum differentiation medium. Death was accompanied by characteristic markers of apoptosis with more than 90% of cells showing DNA fragmentation within 12-16 h. Myoblast death was prevented by IGF-I, des [1–3] IGF-I, IGF-II, and insulin with a dose potency consistent with activation of the IGF-I receptor; death also could be blocked by the protein synthesis inhibitor, cycloheximide. Exogenous IGFs additionally stimulated passage through a single cell cycle and subsequently induced terminal differentiation. Cell survival and cell cycle progression also were enhanced by fibroblast growth factor-2 and platelet-derived growth factor-bb, but these peptides did not promote differentiation. Our results define a novel system for studying apoptotic cell death and its prevention by growth factors, underscore the importance of IGF action in minimizing inappropriate cell death, and indicate that shared signal transduction pathways may mediate myoblast survival in vitro.

The traditional view that growth factors inhibit muscle differentiation (1) has been challenged by recent observations implicating the insulin-like growth factors (IGF-I and IGF-II) in facilitating myoblast differentiation in vitro (2–7), in enhancing muscle growth and regeneration in vivo (8–10), and in modulating muscle mass during fetal development (11, 12). The two IGFs comprise a pair of circulating peptides that are related to each other and to insulin (13). IGF action is initiated by binding to the IGF-I receptor (14–16), a heterotetrameric transmembrane protein that is both structurally similar to the insulin receptor, and uses many of the same intracellular signaling pathways (16, 17). IGF action also is modified by IGF binding proteins (IGFBPs), a family of secreted proteins that bind both IGF-I and IGF-II with high affinity (18, 19). In addition, a number of studies have indicated that the IGF-II receptor, a single-chain transmembrane glycoprotein also known as the cation-independent mannose 6-phosphate receptor and involved in transport of lysosomal enzymes (20), modulates IGF-II action by removing the growth factor from the extracellular environment (14, 20–22).

In previous studies, we and others found that IGF-II is produced by skeletal myoblasts as an early event in their terminal differentiation (2, 23–25) and presented evidence implicating IGF-II as an autocrine differentiation factor (2). Through use of stable C2 cell lines generated to express an IGF-II cDNA in the antisense orientation, we now show that endogenous IGF-II also functions as a critical survival factor during the transition from proliferating to differentiating myoblasts. We have identified IGF-II antisense clones that undergo rapid apoptotic cell death when incubated in low serum differentiation medium. Cell death could be blocked by des [1–3] IGF-I, IGF-I, IGF-II, or insulin with a dose potency appropriate for activation of the IGF-I receptor and also could be prevented by addition of FGF-2 or PDGF-bb to differentiation medium. Our observations thus define a novel autocrine survival role for IGF-II as an early event in muscle differentiation and indicate that shared growth factor signaling pathways may mediate myoblast survival in vitro.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture supplies, fetal bovine serum, newborn calf serum, horse serum, Dulbecco's modified Eagle's medium, Earle's balanced salt solution, and G418 were purchased from Life Technologies, Inc. Plasmid pEMSV scribe2 was a gift from the late Dr. Harold Weintraub (Fred Hutchinson Cancer Center, Seattle, WA), and pSV2neo was from Dr. Paul Berg (Stanford University, Stanford, CA). Recombinant human IGF-I was a gift from Dr. C. A. Morrison (Ciba Geigy, St. Aubin, Switzerland); recombinant human IGF-II and des [1–3] IGF-I were purchased from GroPeP (Adelaide, Australia); recombinant human FGF-2, PDGF-bb, and EGF were purchased from U. S. Biochemical Corp.; insulin, cycloheximide, bisbenzamid, creatine kinase assay reagents, and secondary antibodies were purchased from Sigma. BCA protein quantitation reagents were obtained from Pierce. Restriction enzymes, ligases, and polymerases were purchased from U. S. Biochemical Corp.; Promega Biotech (Madison, WI), New England Biolabs (Boston, MA), and Perkin-Elmer (Norwalk, CT). Radionuclides

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1 The abbreviations used are: IGF, insulin-like growth factor; FGF-2, fibroblast growth factor-2; PDGF-bb, platelet-derived growth factor-bb; EGF, epidermal growth factor; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labeling; FACS, fluorescent activated cell sorting; MES, 2-N-morpholinoethanesulfonic acid; IGFBP, IGF binding protein; des [1–3], desamin 1–3.
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[Fig. 1. Schematic representation of the pEMSVscribble2/IGF-II antisense expression plasmid. The plasmid was constructed as described under "Experimental Procedures." The box represents IGF-II sequences, with the coding region indicated by the hatched box. ATG and TGA codons, the Moloney sarcoma virus promoter (MSV LTR), and simian virus 40 (SV40) polyadenylation sequences are marked by arrows.]

Analysis of DNA Fragmentation by FACS—Cells were grown to ~80% confluency and transferred to differentiation medium in the presence or the absence of 25 nM IGF-I or 1 nM cycloheximide. DNA fragmentation was assessed at 4-h intervals over a 16-h period. After each incubation, cells were trypsinized, resuspended, and washed in phosphate-buffered saline and fixed at ~20 °C in 70% ethanol. Cells were pelleted, washed in phosphate-buffered saline, and resuspended with vortexing in propidium iodide labeling buffer (50 μg/ml propidium iodide, 0.1% sodium citrate, 20 μg/ml ribonuclease A, 0.3% Nonidet P-40, pH 8.3) at 1–5 × 10⁷ cells/ml. Stained cells were stored in the dark at 4 °C until assayed using a Becton Dickinson FACSStar equipped with "Helvit LT" cell cycle analysis software (Ventry software house). Because fixation of cells in ethanol is insufficient to preserve fragmented, low molecular weight DNA inside apoptotic cells, this DNA leaks out during washing and staining, giving rise to the appearance of a pre-G₁ peak, which is considered to be a marker of cell death by apoptosis (37). The extraction of fragmented DNA from apoptotic cells is increased by the addition of phosphate-citric acid buffer (37). The data are expressed as the percentage of total cells in the pre-G₁ phase of the cell cycle.

Analysis of DNA Synthesis—Cells were grown to ~80% confluency and transferred to differentiation medium in the presence or the absence of IGF-I (25 nM), EGF (5 or 50 ng/ml), FGF-2 (10 or 30 ng/ml), PDGF-Mb (2 or 10 ng/ml), or cycloheximide (1 μg/ml). DNA synthesis was assessed by pulsing cells with 10 μM BrdUrd for 4-h intervals over a 24-h period. After each incubation cells were fixed (100% ethanol) and permeabilized (0.25% Triton X-100), and BrdUrd sites were unmasked by incubation with 50 μg/ml of active G418 until 80% confluency was attained. Differentiation was initiated following washing with Earle's balanced salt solution by changing to medium containing Dulbecco's modified Eagle's medium plus 2% horse serum (23). For 2 weeks, medium was changed every 3 days. Twenty colonies were transferred by trypsinization to 12-well cluster dishes and expanded. After screening for production of chimeric mRNA, two colonies were selected for further characterization.

Cell Culture—Transfected cells were routinely plated at 100,000 cells/ml on gelatin-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum, 10% heat-inactivated fetal bovine serum, and 400 μg/ml active G418 until 80% confluency was attained. Differentiation was initiated following washing with Earle's balanced salt solution by changing to medium containing Dulbecco's modified Eagle's medium plus 2% horse serum (23). Twenty colonies were counted in a hemocytometer after trypsinization; alternatively, nuclei were counted after cells were fixed and stained with bisbenzamide. The number of detached cells was determined by counting an aliquot of culture medium in a hemacytometer. Cell viability was established by replating detached cells in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum, 10% heat-inactivated fetal bovine serum, and 400 μg/ml active G418.

RNA Isolation and Analysis—Total RNA was isolated from cells using a modified guanidinium thiocyanate method (29) and quantitated by spectrophotometry. RNA integrity was assessed by electrophoresis through 1% agarose, 2.2 M formaldehyde gels after staining with ethidium bromide. Solution-hybridization ribonuclease protection assay was performed using a 32P-labeled riboprobe synthesized in vitro (32) using a linearized plasmid template and T7 RNA polymerase.

IGF-II Radiolmmunossay—Conditioned differentiation medium (Dulbecco's modified Eagle's medium plus 2% horse serum) was harvested, clarified by low speed centrifugation, and stored at ~20 °C until assayed. IGF-II concentrations were determined by radiodniunoassay following acid ethanol precipitation (33, 34). Recombinant human IGF-II was used as standard and tracer in an equilibrium assay established with a monoclonal anti-rat IGF-II antibody at 2.5 ng/tube (35). IGF-II was used as standard and tracer in an equilibrium assay established with a monoclonal anti-rat IGF-II antibody at 2.5 ng/tube (35). Recombinant human IGF-II was used as standard and tracer in an equilibrium assay established with a monoclonal anti-rat IGF-II antibody at 2.5 ng/tube (35).

Creatine Kinase Assay—Cytoplasmic lysates were collected from differentiating cells by incubation with 50 mM Tris-MES, pH 7.8, 1% Triton X-100 for 10 min at 25 °C. Samples were stored at ~80 °C and assayed within 1 week of collection using a commercially available kit (Sigma). Enzymatic activity was normalized to total protein content as determined by the BCA protein assay.

TUNEL Assay for DNA Fragmentation—Cells were grown to ~80% confluency and induced to differentiate as described. After 12 h, cells were fixed (100% ethanol), and DNA was labeled by treatment with terminal deoxynucleotidyl transferase and biotinylated dUTP followed by incubation with avidin fluorescein isothiocyanate. If biotinylated dUTP is enzymatically added to available 3'-OH ends of DNA, it is then detected by the addition of avidin/flourescein isothiocyanate followed by fluorescence microscopy. The results are expressed as the percentage of total cells with fluorescent nuclei.

Both antisense lines displayed a rapid decline in the number of adherent, viable myoblasts following transfer into differentiation medium with a half-life of 15.5-16 h.2

In C2 cells and other myoblast lines, IGF-II secretion accompanies differentiation (2, 23, 24). To verify the effectiveness of the antisense transgene in blunting IGF-II expression, IGF-II levels were measured by radiodniunoassay in conditioned differentiation medium from both cell lines. The values were consistently less than or equal to levels found in nonconditioned medium (≤1.2 ± 0.2 nm), thus indicating that the antisense approach was successful in inhibiting growth factor expression.

2 C. E. H. Stewart and P. Rotwein, unpublished observations.
tiation medium. Only 50% of cells from antisense line 12 remained attached to the culture dish by 24 h, and only 15% remained by 72 h. A slower fall in cell number was seen with line 3 (Fig. 3). For both lines, cell death was comparable when myoblasts were incubated in differentiation medium containing 0–2% horse serum. Nuclear staining using the dye bisbenzamide showed many cells with condensed nuclei, and analysis of chromosomal DNA extracted from detached cells revealed DNA laddering, indicating that IGF-II antisense cells were undergoing apoptotic cell death when incubated in differentiation medium. In addition, detached cells were incapable of reattaching to culture dishes when incubated in growth medium containing 20% serum.

IGF-I and Cycloheximide Prevent Premature Myoblast Death—The addition of IGF-I or cycloheximide to differentiation medium blocked cell death in IGF-II antisense lines 3 and 12. As shown in Fig. 4A, the dramatic decline in the number of adherent cells in antisense line 12 during a 24-h incubation in differentiation medium was prevented by 1 μg/ml cycloheximide or 25 nM IGF-I. Treatment with cycloheximide completely blocked cell death for at least 36 h in both antisense lines, and incubation with IGF-I led to a 25–50% increase in the number of adherent myoblasts (Fig. 4B).

Treatment of IGF-II antisense 12 cells with IGF-I also prevented DNA fragmentation. As assessed by TUNEL assay (Fig. 5A), over 90% of untreated antisense 12 myoblasts incorporated biotinylated dUTP into their nuclei following 12 h in differentiation medium, whereas less than 10% of IGF-I-treated cells were labeled. A similar decline in the number of labeled nuclei was seen when antisense cells were treated with cycloheximide. FACS analysis performed on antisense 12 cells treated with IGF-I or cycloheximide confirmed results obtained with the TUNEL assay. Untreated antisense cells showed a marked increase in the percentage of cells showing DNA fragmentation, from ~30% at 0 h to ~95% at 16 h. By contrast, only 15–25% of cycloheximide-treated cells displayed DNA fragmentation during the same intervals, whereas fewer than 10% of IGF-I treated cells had a similar pre-G1 apoptotic peak (Fig. 5B).

IGF-I Promotes DNA Synthesis and Cell Replication in IGF-II Antisense Myoblasts—The results shown in Figs. 4 and 5 indicated that IGF-I treatment inhibited apoptotic cell death and increased the total number of viable cells. To assess the mechanisms leading to this rise in cell number, we examined the effects of IGF-I on DNA synthesis. As shown in Fig. 6A, incubation of IGF-II antisense 12 myoblasts with differentiation medium containing IGF-I increased incorporation of the nucleotide analog BrdUrd into chromosomal DNA. More than...
60% of IGF-I-treated myoblasts became labeled during the last 4 h of an 8- or 12-h incubation with growth factor. 35% of cells were labeled during the last 4 h of a 16-h treatment, but fewer than 10% were labeled following a 20- or 24-h incubation with IGF-I. By contrast, the fraction of cells entering S phase declined dramatically in antisense myoblasts incubated in differentiation medium alone, dropping from 50% during the first 4 h to 5% by 12-16 h. As expected, cycloheximide treatment blocked progression into S phase, and few nuclei incorporated BrdUrd (Fig. 6B). Similar results were seen when experiments were performed using serum-free differentiation medium.\textsuperscript{2}

Ligand-induced Activation of the IGF-I Receptor Mediates Survival of IGF-II Antisense Myoblasts—Fig. 7 shows a series of dose-response curves examining myoblast survival following...
a 24-h incubation in differentiation medium containing graded concentrations of IGF-I, IGF-II, the IGF-I analog des [1–3] IGF-I, or insulin. At the highest doses used, all four growth factors inhibited myoblast death and stimulated replication, as indicated both by a decline in the fraction of detached dead cells and a rise in the number of adherent myoblasts. Des [1–3] IGF-I was the most potent agent, with an ED50 of approximately 0.5 nM, followed by IGF-I (~8 nM), IGF-II (~20 nM), and insulin (~500 nM). These dose-response curves are consistent with mediation of cell survival and proliferation by the IGF-I receptor.

Apoptotic Cell Death Is Prevented in IGF-II Antisense Cells by FGF-2 and PDGF-bb—The addition of FGF-2 or PDGF-bb to the differentiation medium blocked cell death of antisense line
12. As shown in Fig. 8, the dramatic decline in the number of adherent cells during a 24-h incubation in differentiation medium was prevented by 10 or 30 ng/ml FGF-2 or 10 ng/ml PDGF-bb. By contrast, EGF at both doses tested (5 or 50 ng/ml) and PDGF-bb at its lower dose (2 ng/ml) were incapable of preventing myoblast death. Similar rescue profiles were observed when the number of detached, dead cells were counted (Fig. 8). Treatment of antisense 12 cells with FGF-2 or PDGF-bb but not EGF also prevented DNA fragmentation as assessed by TUNEL assay.2

FGF-2 and PDGF-bb Promote DNA Synthesis and Cell Replication but Not Differentiation in IGF-II Antisense Myoblasts—The results shown in Fig. 8 indicated that FGF-2 and PDGF-bb inhibited apoptotic cell death and enhanced the number of surviving cells by 75–100%. To address whether the rise in cell number was attributable to enhanced cell replication, we monitored progression through S phase of the cell cycle by assessing BrdUrd uptake into DNA. As shown in Fig. 9, treatment of antisense 12 cells with BrdUrd for the last 4 h of a 12-h incubation with either growth factor resulted in labeling of 40–60% of the cells. By contrast, EGF had little effect at either dose tested (5 or 50 ng/ml). As demonstrated with IGF-I treatment, the fraction of cells entering S phase dropped dramatically following a 24-h incubation with FGF-2 or PDGF-bb, although 15–20% of the cells still incorporated BrdUrd.

As seen in Fig. 10, treatment of antisense 12 cells with a single dose of IGF-I at the onset of incubation in differentiation medium resulted in measurable creatine kinase activity by 72 h. Creatine kinase values of ~2500 milliunits/mg of total protein were obtained, similar to those seen in nontransfected and nontreated differentiating C2 cells at 72 h.2 By contrast, enzymatic activity in FGF-2- or PDGF-bb-treated antisense myoblasts was at least 20-fold lower and was equivalent to values seen when C2 cells were incubated in growth medium.2 In addition, myotubes were seen only in IGF-I-treated cells.2

**DISCUSSION**

Previous studies have documented roles for IGF-I and IGF-II in stimulating myoblast proliferation and differentiation in cell culture (reviewed in Ref. 3) and in enhancing muscle mass in vivo (10–12). Cultured myoblasts have been shown to express IGF-II mRNA and protein as an early event in differentiation (2, 23–25), and several lines of evidence have implicated IGF-II as an autocrine differentiation factor (2, 38). In this report, we show that IGF-II also acts as an autocrine survival factor for myoblasts during the transition from proliferating to differentiating cells. By neutralizing IGF-II expression through stable transfection of C2 myoblasts with an expression plasmid containing a mouse IGF-II cDNA in the antisense orientation, we have identified cell lines that undergo rapid apoptotic cell death when cultured in low serum differentiation medium. Myoblast death could be prevented by the addition of IGF-I, des [1–3] IGF-I, IGF-II, or insulin to the medium with a dose potency consistent with activation of the IGF-I receptor. Cell death also could be blocked by FGF-2 and PDGF-bb, indicating that shared growth factor signaling pathways may mediate myoblast survival in this system.

In previous studies using antisense oligonucleotides to IGF-II mRNA, we found that IGF-II was needed for terminal differentiation of C2 cells but did not appear to be required for cell survival (2). This apparent discrepancy between past and current observations may reflect differences in experimental design. Because the oligonucleotides were added to the incubation medium at the onset of differentiation, it is possible that some IGF-II mRNA and protein were produced prior to inhibition of gene expression. In other experiments, Montarras et al. (38) generated C2 cell lines expressing an antisense IGF cDNA and showed that these cells differentiated poorly, again confirming the role of endogenous IGF-II in myoblast differentiation. Similarly, we have identified IGF-II antisense lines that survive in low serum differentiation medium but do not differentiate, and preliminary experiments suggest that these cells maintain low level secretion of IGF-II.2 Taken together, these results indicate that IGF-II is required for C2 cell survival in the absence of other growth factors.

IGF-mediated myoblast survival was accompanied by stimulation of cell proliferation, as indicated by enhanced entry into
S phase of the cell cycle and by increased cell number. This proliferative effect appeared to be limited to progression through a single cell cycle, because the fraction of myoblasts in S phase as measured by incorporation of BrdUrd into DNA over 4-h intervals dropped precipitously after 16–20 h and total cell number rose only by a factor of two. Longer incubations with IGF-I led to induction of myoblast differentiation, so that by 72 h myotubes were evident, and creatine kinase activity was increased. This temporal progression from proliferating to terminally differentiated myoblasts has been described previously for rat L6E9 and L6A1 cell treated with IGF-I or IGF-II (5, 39, 40), although in the latter cell line it has been suggested that IGF-II only stimulates differentiation, whereas IGF-I promotes both replication and differentiation (40). By contrast, in C2 myoblasts, we found that des[1–3]IGF-I, IGF-I, IGF-II, and insulin all could promote passage through one cell cycle and subsequent differentiation, with a dose potency reflecting affinity for the IGF-I receptor. Because des[1–3]IGF-I binds poorly to IGFBP-5, the single IGFBP produced by C2 myoblasts (41), our results additionally support a role for this IGFBP in inhibiting IGF action in muscle cells. The differences between our observations and those of Ewton et al. (40) thus may indicate variability in IGF-I receptor number or in types of IGFBPs expressed by C2 and L6A1 cells, respectively.

IGF-I and IGF-II have been shown to function as survival factors for several other cell types (42, 43). In cultured cerebellar granular neurons, IGF-I prevented cell death induced by low levels of potassium (42). Other growth factors, including FGF-2 and PDGF, were ineffective (42), in contrast to our results with C2 myoblasts. IGF-II has been identified as the growth factor required for full tumorigenesis in transgenic mice expressing simian virus 40 T antigen in the islets of Langerhans (44). In the absence of IGF-II action, these cells show an enhanced rate of death, and tumor formation is reduced (44). IGF-I and PDGF have been found to blunt apoptosis induced by c-Myc in serum-deprived fibroblasts, an effect that does not require cell cycle progression or ongoing protein synthesis (45). In other experiments, IGF-I and the IGF-I receptor were shown to be required for survival of cultured hematopoietic cells after trophic factor withdrawal (46) to prevent apoptosis.
tosis in fibroblasts exposed to the topoisomerase inhibitor, eto-
poside (45, 47), and to block the death of a variety of tumor cell
lines cultured for short term in vivo (43, 48). One general
conclusion that emerges from these various observations is
that IGF action can prevent the premature death of many cell
types, a conclusion supported by the marked cellular hypopla-
sia in tissues of mice lacking a functioning IGF-I receptor (12).

Recent observations have indicated a role for phosphatidylinosi-
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IGF-II can block the rapid death of primary skeletal myoblasts
isolated from mice with muscular dystrophy (52), elucidation of
the signal transduction pathways responsible for these actions
may have important clinical implications.

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