Differentiation in C₂C₁₂ myoblasts depends on the expression of endogenous IGFs and not serum depletion

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Yoshiiko, Yuji, Keita Hirao, and Norihiko Maeda. Differentiation in C₂C₁₂ myoblasts depends on the expression of endogenous IGFs and not serum depletion. Am J Physiol Cell Physiol 283: C1278–C1286, 2002; 10.1152/ajpcell.00168.2002.—Myogenic differentiation in vitro has been usually viewed as being negatively controlled by serum mitogens. A depletion of critical serum components from medium has been considered to be essential for permanent withdrawal from the cell cycle and terminal differentiation of myoblasts. Removal of serum mitogens induces the expression of insulin-like growth factors (IGFs), whereas it inhibits that of basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β in myoblasts. These responses of growth factors to medium conditioning seem to be well matched to their functions in proliferation/differentiation. In the present study, we showed that C₂C₁₂ myoblasts differentiated actively, even in mitogen-rich medium, and that this medium offered an advantage over mitogen-poor medium in terms of increasing differentiation. Our attention focused on endogenous growth factors, as described above, especially IGFs in mitogen-rich medium. During differentiation, IGF-I and IGF-II mRNA levels increased, but bFGF and TGF-β₁ mRNAs decreased. Differentiation was commensurable with IGF mRNA levels and suppressed by antisense oligodeoxynucleotides and neutralizing monoclonal antibodies against IGFs. These results suggest that an autocrine/paracrine loop of IGFs, bFGF, and TGF-β₁ is active in proliferating and differentiating C₂C₁₂ cells without a depletion of serum and that endogenous IGFs actively override the negative control of differentiation by serum mitogens.

myoblasts; insulin-like growth factor I; insulin-like growth factor II; serum mitogens; differentiation

PROLIFERATION AND DIFFERENTIATION in myoblast cultures are subject to control by serum mitogens, as demonstrated by effects of medium conditions or mitogen addition or removal. The majority of serum mitogens are considered to act as an inhibitor of myogenic differentiation via, at least in part, the downregulation of myogenic determination factors (4, 11, 22, 26, 30, 36, 38, 50, 57). The most extensively studied myogenic and/or myogenic factors are basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β, and insulin-like growth factors (IGFs; see Ref. 14).

These studies, using nearly confluent myoblasts, were conducted under mitogen-free or -poor conditions. Both bFGF and TGF-β act as potent inhibitors of myogenic differentiation, whereas bFGF stimulates myoblast proliferation (25, 33) but TGF-β does not (25). TGF-β is also unique, because it favors myoblast differentiation in mitogen-rich medium (61). IGFs have the unusual property of stimulating both proliferation and differentiation of myoblasts under serum-starved conditions (12, 13, 45). IGF-I activates proliferation first and subsequently stimulates muscle-specific gene expression (12, 13, 45), and it is more active in stimulating proliferation but less active in stimulating differentiation than IGF-II (13).

Without endogenous agents, mitogen-poor conditions cause differentiation and subsequent expression of IGF-I and IGF-II in myoblasts such as the C₂C₁₂ line (6, 9, 17, 34, 43, 44, 48, 49, 52, 53), and differentiation correlates with IGF mRNA levels in myoblast sublines (17, 43). This finding was also confirmed by overexpression (48) and antisense (16, 17, 34, 52) studies. In contrast to their low levels of IGFs, proliferating myoblasts express FGF (2, 20, 35) and TGF-β (5, 29) at high levels, and a depletion of serum was shown to be required for their downregulation (2, 20, 29, 35). Sol 8 cells transfected with the FGF antisense expression vector differentiated faster than control cells and showed increased levels of myogenin mRNA in response to a depletion of serum (19). Given these observations, most investigators in this field believe that a depletion of serum is essential for myogenic differentiation in vitro and that IGFs have a potential role in the proliferation/differentiation process. However, myogenic differentiation in vitro has been commonly examined under conditions different from those present in the environment of developing tissues, where mitogens presumably abound (61).

In the present study, we obtained solid evidence that confluent C₂C₁₂ myoblasts differentiated actively, even in a mitogen-rich medium. Unexpectedly, the medium offered an advantage over mitogen-poor medium in terms of increasing differentiation. In this model, we found the systematic upregulation of the expression of...
IGF genes and downregulation of that of bFGF and TGF-β1 genes without a depletion of serum. From these results, we hypothesize that endogenous IGFs can override the negative control of differentiation by serum mitogens.

MATERIALS AND METHODS

Cell cultures. The C2C12 cell line, which is a subclone of the C2 myoblast line isolated from satellite cells of adult mice (58), was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% FBS (Upstate Biotechnology, Lake Placid, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin [mitogen-rich, growth-promoting medium (GM)]. The cells were plated at 6 × 10^5/cm² in tissue culture plates or dishes. The medium was renewed or changed to DMEM supplemented with 1% FBS [mitogen-poor, differentiation-promoting medium (DM)] when the cells reached 90% confluence. All cultures were incubated at 37°C in an atmosphere of 95% humidity and 5% CO₂. Both types of medium were changed every second day.

Quantification of differentiation. The creatine kinase (CK) activity in cell lysates, which indicates myogenic differentiation over the culture period, as described previously (13), was determined. Cells were rinsed with PBS, stored at −80°C in cell lysis buffer (1 M NaCl, 1 mM EDTA, 1% Triton X-100, and 10 mM Tris·HCl, pH 7.2), and thawed on ice before the assay (24). Lysates were centrifuged at 20,000 g for 10 min at 4°C. CK activity in aliquots of the supernatant was determined with a commercial assay kit (Wako, Tokyo, Japan). In addition, cultures to be scored for myotube formation were washed with PBS, fixed in methanol, and stained with Giemsa. The percentage of nuclei in myotubes was estimated by counting a total of 1,200–1,500 nuclei in randomly selected fields under a microscope as described previously (26). Cell morphology was determined in the same fields.

DNA synthesis. Cells were treated with 5-bromo-2’-deoxyuridine (BrdU) for 2 h before culture termination, and BrdU-positive cells were detected immunocytochemically with mouse anti-BrdU antibody (PROGEN Biotechnik, Heidelberg, Germany) and horseradish peroxidase-conjugated goat anti-mouse IgG (ZYMED Laboratories, San Francisco, CA) as the second antibody. The fraction of cells with BrdU-positive nuclei was determined by counting over 1,000 nuclei in randomly selected fields under a microscope.

RNA preparation and RT-PCR analysis. Total cellular RNA was extracted from each culture by the acid guanidinium thiocyanate single-step protocol (8). cDNA was synthesized from 1 μg of total RNA by using random hexamers as a primer and amplified from 20 to 45 cycles with a RNA PCR kit (Takara, Kusatsu, Japan). Primer sets for IGF-I, IGF-II (51), bFGF, TGF-β1, (23), myogenin (42), the γ-subunit of nicotinic ACh receptor (γ-AChR; see Ref. 42), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Ref. 48) were designed as described. For quantitative analysis of IGF-I, IGF-II, myogenin, and γ-AChR mRNAs, products were electrophoresed on 1.5% agarose gels and visualized by staining with ethidium bromide. The luminescence was determined with a charge-coupled device image sensor (ATTO, Tokyo, Japan). For determination of initial levels of templates, the regression equation y = a × b^n, where y is the luminescence and n is the number of cycles, was fitted to the data in the linear portion of semilogarithmic graphs (59). The constants a and b of the equation are the amounts of original templates and the efficiency of amplification for each cycle, respectively. For analysis of bFGF and TGF-β1 mRNAs, 26–30 cycles of amplification were used, and the products were resolved on agarose gels as described above. GAPDH was used as an internal control.

Preparation of digoxigenin-labeled riboprobes. Riboprobes were synthesized and labeled with digoxigenin (DIG)-11-UTP by in vitro transcription. We subcloned mouse IGF-I (PMIGF1–2, ATCC no. 63070) and IGF-II (PMIGF2–3, ATCC no. 63071) cDNA into the plasmid vector pSPT18. The method for the DIG-labeled riboprobe synthesis was as recommended by the manufacturer, which involved the use of a nonradioactive RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). The efficiency of riboprobe labeling was estimated by comparison with the DIG-labeled control riboprobe. To analyze the specificity of riboprobes, we prepared DNA dot blots of templates and control plasmid.

Northern blot analysis. Aliquots of RNA (10 μg) were electrophoresed on 1% denatured formaldehyde-agarose gels, transferred to positively charged nylon membranes (Boehringer Mannheim), and immobilized by baking at 120°C for 30 min. Membranes were then incubated in prehybridization buffer (500 ml) at 68°C and hybridized with DIG-labeledriboprobes overnight at the same temperature. After the washing procedure, alkaline phosphatase-conjugated F(ab)² fragments of anti-DIG antibody were applied, and chemiluminescence detection was then performed by using di zona 3-[(4-methoxyxyspiro[1,2-dioxetane-3,2’(5’-chloro)tricyclo[3.1.1.1^3,7]decan]-4-yl)phenyl phosphate as the substrate (Boehringer Mannheim). Hybridization was determined by using Kodak scientific imaging films (BIOMAX MS, Rochester, NY). After hybridization with IGF probes, membranes were stripped and rehybridized with the β-actin riboprobe as an internal control.

Antisense oligodeoxynucleotide treatment. The 20-mer antisense oligomers (ASO) for IGF-I and IGF-II, which complemented the initial coding regions of mouse IGF-I and IGF-II, respectively (40), and their corresponding sense (SO; see Ref. 40) and additional control (CO; see Ref. 17) oligomers were synthesized by the phosphorothioate approach with tetrathyliuram disulfide. Each oligomer for IGF-I was mixed with an equal concentration of its corresponding oligomer for IGF-II and enclosed within positively charged liposomes (Tfx-50; Promega, Madison, WI). After the cells had reached 90% confluence, the cultures were supplied daily with fresh GM, including oligomer-liposome mixtures or liposomes alone, and were incubated three more days. CK activity and myotube formation were determined as described above. The effect of oligomer-liposomes or liposomes alone (final oligomer concentration of 1.5 μM for IGF-I and 1.5 μM for IGF-II) and Tfx-50 (0.3%) on the production of IGF proteins by cultures treated with the agents for 2 days was assessed by the immunoperoxidase antibody method. Briefly, after the treatment, the cells were fixed in periodate-lysine-parafomaldehyde for 10 min and washed with PBS. After freezing and thawing, the cells were immunocytochemically stained with anti-IGF-I (mouse anti-human IGF-I, IgG1; Upstate Biotechnology) or anti-IGF-II (mouse anti-rat IGF-II, IgG1; Upstate Biotechnology) monoclonal antibody and visualized with a Vectastain avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). Toxicity of oligomers and liposomes was evaluated by using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or by trypan blue staining of cells.

Treatment with neutralizing monoclonal antibody. A mixture of equal amounts of anti-IGF-I and -IGF-II (25–100 μg/ml) or control mouse IgG1 (100 μg/ml; Chemicon International, Temecula, CA) was added to cultures along with fresh GM when the cells had reached 90% confluence. The cultures
were incubated further for 3 days and then used to determine CK activity and myotube formation, as described above. Statistical analysis. Statistical evaluations were made by ANOVA with multiple comparisons done by the method of Fisher's protected least-significant difference. Significance of differences was accepted at \( P < 0.05 \).

RESULTS

Comparison of differentiation between DM and GM cultures. First, we determined whether there would be phenotypic differences between cells in mitogen-poor DM and mitogen-rich GM cultures. C2C12 cells were used because of their ability to differentiate rapidly and produce IGFs in response to a depletion of serum. Figure 1 compares effects of DM and GM media on CK activity and myotube formation. As previously reported (17), CK activity increased immediately after the cultures had been exposed to DM, peaked on the third day, and subsequently decreased over time. Similarly, cells in GM cultures showed a marked increase in CK activity. The increase lagged a little behind that in DM cultures, but the maximum level became significantly higher than that in the DM cultures. The activity then remained high throughout the remaining culture period. Consistent with this result, the percentage of myotube formation in GM cultures was larger than that in DM cultures, and the high percentage

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Fig. 1. Comparison of creatine kinase (CK) activity and myotube formation by C2C12 myoblasts between growth medium (GM) and differentiation medium (DM) cultures. Cells were initially grown in GM. When the cells reached 90% confluence (day 0), the medium was renewed (● and filled bars) or changed to DM (○ and open bars). Media were changed every 2nd day. At the times indicated, CK activity in cell lysates was determined, and other cultures were subjected to scoring of myotube formation under a light microscope. Results for CK activity are the means ± SD from 4 samples. *\( P < 0.05 \) and **\( P < 0.01 \), significant difference from maximum level on day 3 in DM.

Fig. 2. Differentiation of C2C12 myoblasts in GM cultures. Cells were grown in GM as described in Fig. 1. A: morphology of cells as visualized with a phase-contrast microscope. The end of the proliferative phase of cells (a) and multinucleate myotubes formed at the 4th day after cells had reached 90% confluence (b) are shown. Magnification: ×95. B: relationship between DNA synthesis and CK activity in GM cultures. Cells were incubated with 10 mM 5-bromo-2'-deoxyuridine (BrdU) for 2 h before culture termination, subsequently fixed, and immunostained with mouse anti-BrdU antibody and horseradish peroxidase-conjugated secondary antibody. The fraction of myoblasts with BrdU-positive nuclei was determined under a light microscope. Cells for CK activity were cultured in parallel to those for BrdU incorporation. Results for CK activity are the means ± SD from 4 samples.
continued up to the time of culture termination, whereas a delay in myotube formation was observed in the former cultures (Fig. 1). As a consequence, GM cultures gave well-developed myotubes (Fig. 2A) and an increase in CK activity in contrast to a decrease in the number of BrdU-positive cells (Fig. 2B).

PCR analysis of myogenic differentiation markers and growth factors in GM cultures. After we had characterized morphological and biochemical properties of C2C12 cell differentiation in GM cultures, our attention was next drawn to the early molecular consequences of this cellular process. Using the quantitative PCR method, we determined the levels of myogenin, γAChR, and IGF mRNAs in proliferating myoblasts (Fig. 3). Initial levels of templates were calculated by the formula described in MATERIALS AND METHODS. As shown in Fig. 3A, by 24 h, no mRNA had changed in level compared with that in the proliferating myoblasts (0 h). By 36 h, however, the levels of myogenin and IGF-I mRNAs had increased 2.5- and 3-fold, respectively, and had markedly increased by 48 h (5- and 9-fold increases for myogenin and IGF-I, respectively). Levels of IGF-II and γAChR mRNAs were unchanged by 36 h, although they did increase by 48 h (6- and 4.5-fold increases for γAChR and IGF-II, respectively). PCR products for bFGF and TGF-β1 mRNAs were found in proliferating myoblasts, but their levels decreased gradually by 72 h (Fig. 3B). The pattern of their mRNA levels was not identical, i.e., bFGF mRNA could not be detected after 36 h, but only a slight level of TGF-β1 mRNA was observed until 48 h.

Comparison of IGF mRNA accumulation between DM and GM cultures. By quantitative PCR analysis, we observed that C2C12 cells began active IGF-I and IGF-II gene expression without a depletion of serum. So we next examined the expression pattern of these two genes in relation to differentiation in DM and GM media. Total RNA was produced in parallel incubations in these media, and Northern blot analysis was performed. As shown in Fig. 4, blots hybridized with specific riboprobes for mouse IGF-I and IGF-II revealed single-sized transcripts. In rodents, IGF genes are transcribed from various promoters; but, as judged by Northern blot analysis in differentiating C2 cells, single-sized transcripts were observed for both IGF-I and IGF-II (6, 17, 28, 43, 49, 52, 53). As described elsewhere, we could not find IGF-I and IGF-II mRNA in proliferating myoblasts. However, their transcripts were found from day 2 under both conditions, and the pattern of their expression was different between the two genes in relation to differentiation in DM and GM media.
two cultures. In DM cultures, levels of IGF-I and IGF-II mRNA increased transiently, having a peak on days 2 and 5, respectively, and started to decrease by the eighth day. In contrast, GM caused a relatively slow but steady increase in these mRNA levels during the culture period.

Effects of ASO and neutralizing monoclonal antibodies against IGF-I and IGF-II on CK activity and myotube formation in GM cultures. Possible autocrine/paracrine effects of a protein are directly related to protein production rather than to gene expression. To identify the contribution of IGF proteins to differentiation in GM cultures, we prepared an equivalent mixture of ASO for mouse IGF-I and IGF-II mRNA and introduced it in cells via liposomes with fresh medium daily to facilitate the transfection and effects of ASO in the presence of serum. First, we determined the effect of the oligomers on IGF protein production immunocytochemically (Fig. 5A). Cells treated with SO or CO (data not shown) were immunoreactive with anti-IGF-II. However, no immunoreaction was observed in ASO-treated cells. The same result was obtained from immunocytochemistry for IGF-I (data not shown). Also, we demonstrated no toxicity of liposomes and oligomers at concentrations of 0.3% and 1.5 μM for IGF-I plus 1.5 μM for IGF-II, respectively (data not shown). As shown in Fig. 5B, although the addition of SO, CO, or liposomes alone (data not shown) had no effect on

Fig. 5. Effect of oligomers against IGFs on C2C12 myoblast differentiation in GM cultures. After the cells had reached 90% confluence (day 0), antisense (ASO) or control [sense (SO) and control (CO) oligomer] oligomer-liposome mixtures or liposomes alone were added to the cultures with fresh GM, and the cultures were incubated further for 2–3 days. The medium containing oligomers and/or liposomes was changed daily. A: immunocytochemical detection of IGF-II in oligomer-treated cells. IGF-II was determined immunocytochemically by using anti-IGF-II monoclonal antibody and the avidin-biotin complex system. Cells incubated with SO (a) or ASO (b) for 2 days are shown as visualized under a light microscope at ×95 magnification. B: effects of oligomers on CK activity and myotube formation. Cells were incubated with ASO, SO, or CO for 3 days. Results for CK activity are the means ± SD from 4 samples. **P < 0.01, significant difference from liposomes (Tfx-50) alone. C: effect of oligomers on the morphology of cells. Cells treated with ASO (a) or with SO (b) are shown as visualized by phase-contrast microscopy at ×95 magnification.
CK activity or myotube formation, ASO significantly decreased the values of these parameters. Phase-contrast microscopy revealed that myotubes formed in the presence of ASO were relatively smaller and round in shape (Fig. 5C).

To further confirm the role of IGFs in C2C12 cell differentiation in GM cultures, we also determined the effects of neutralizing anti-IGF-I and -IGF-II antibodies on CK activity and myotube formation. As shown in Fig. 6, 50 and 100 μg/ml anti-IGF antibodies significantly reduced not only CK activity but also myotube formation. The values of these parameters did not change in the presence of 100 μg/ml control IgG1. The morphology of C2C12 cells treated with anti-IGF-I and -IGF-II antibodies was essentially the same as that seen in the ASO experiment (data not shown).

**DISCUSSION**

A depletion of serum from myoblast cultures has been considered to be essential for terminal differentiation, because serum mitogens generally inhibit differentiation and/or stimulate proliferation (4, 11, 22, 25, 26, 30, 33, 36, 38, 50, 57, 61). In addition to bFGF and TGF-β, interferon-β (36), platelet-derived growth factor-BB (26, 57), acidic FGF (30, 38), leukemia inhibitory factor, TGF-α, interleukin-6 (4), keratinocyte FGF (38), tumor necrosis factor (50), and hepatocyte growth factor (22) have been shown to inhibit differentiation and/or stimulate proliferation. A depletion of serum is also responsible for the induction of IGFs in myoblasts (6, 9, 34, 35, 43, 48, 49, 52, 53). This is supported by the study showing that the addition of serum to subconfluent L6 cells decreased the level of IGF-II mRNA and also that TGF-β, IGF, and insulin in the serum acted as an inhibitor of the accumulation of IGF-II mRNA (31).

These observations do not seem to be in conflict with our result showing a small lag in the induction of differentiation in the GM cultures. However, we demonstrated that the mitogen-rich medium predominated eventually over the mitogen-poor one in terms of increasing differentiation. The stimulatory effect of mitogen-rich medium on C2C12 cell differentiation does not seem to be due to the successive stimulation for proliferation, because the cells lost their DNA synthesis dramatically with increasing differentiation. Given these findings, it is noteworthy that cellular conditions in mitogen-rich medium probably differ from those in mitogen-poor medium. Several studies suggest that certain serum components, such as retinoic acid (1, 3, 21), thyroid hormone (1, 7, 37), glucocorticoid (56, 60), and neuregulin (18), are potent stimulators of myogenic differentiation in L6 and C2 cells. Interestingly, some of them, i.e., retinoic acid (31) and glucocorticoid (31, 56, 60), may play a role as a stimulator of differentiation via the IGF-II signaling pathway.

It is estimated, based on human (41) and rat (54) data, that mitogen-rich medium may contain approximately <100 ng/ml IGF, which is capable of stimulating differentiation in mitogen-free or -poor medium but may be insufficient to stimulate differentiation in mitogen-rich medium. This hypothesis is supported by an earlier study showing that differentiation of L6 cells required treatment with >100 ng/ml IGF in the presence of serum (15). Therefore, our results suggest that C2C12 cells are not adequately supplied with IGFs in mitogen-rich medium and that autocrine/paracrine IGFs actively override the inhibitory effect of serum mitogens on differentiation in collaboration with retinoic acid (31), glucocorticoid (31, 56, 60), and so on.

Thus there is no necessity for a depletion of serum from the medium to upregulate IGF-I and IGF-II expression in C2C12 cells. This is also true in the case of bFGF and TGF-β1. The expression of FGFs (2, 20, 35) and TGF-β (5, 29) in myoblasts is upregulated by serum, quite opposite to the case of IGFs (6, 9, 17, 34, 43, 44, 48, 49, 52, 53). The expression of FGF genes (bFGF and acidic FGF) in mouse Sol 8 and rat L6 myoblasts decreased markedly during differentiation in mitogen-poor medium (2, 20). A similar situation appears to exist for another major inhibitor of myogenic differentiation, TGF-β1 (5, 29); a decrease in TGF-β1 mRNA levels (but increases in TGF-β2 and TGF-β3) in differentiated C-2 cells was demonstrated under mitogen-poor conditions (29).

We have no evidence indicating the mechanism for a given pattern of growth factor expression during the C2C12 cell proliferation and differentiation. Possible explanations of this automatism in gene expression can be considered from the following observations: MyoD-expressing P19 pluripotent embryo carcinoma cells differentiate into skeletal muscle, which indicates that differentiation may be modulated by cell-to-cell interaction during cell aggregation (47). This is supported by the result showing that avian embryonic myoblast fusion was inhibited by a synthetic peptide that contained the H-A-V sequence or by monoclonal
anti-N-cadherin antibody (32) and that the interaction of avian muscle cells with collagen type I matrix facilitated the release of IGF-I and IGF-binding proteins (39). Taking these observations together, we suggest that the cells’ reaching confluence and subsequent cell-to-cell and cell-to-matrix interaction are important to upregulate IGFs and facilitate differentiation and that bFGF and TGF-β1 are downregulated and deprived of their effects with increasing differentiation but upregulated and facilitate rapid growth of the cells during proliferation.

TGF-β1 induces a rapid decrease in c-myc expression and favors myoblast differentiation with permanent withdrawal from the cell cycle in mitogen-rich medium (61). We demonstrated here that endogenous TGF-β1 was downregulated during differentiation but that some expression remained up to 48 h after 90% confluence. Treatment of cells with IGFs can downregulate the expression of TGF-β1 (5), and IGF expression can also be downregulated by TGF-β (31). These observations seem to be compatible with each other.

IGF-I induces myogenin expression via type I IGF receptor-mediated signaling, which is also responsive to IGF-II (16). Two studies indicate that IGF-II mRNA is expressed after the appearance of myogenin mRNA (6, 43) but before that of contractile protein mRNA (43). We also confirmed this sequential gene expression in mitogen-rich medium, i.e., IGF-I mRNA increased at the same time or before myogenin mRNA, whereas an increase in IGF-II mRNA came after that of myogenin and IGF-I. Although the difference between IGF-I- and IGF-II-mediated functions is still under investigation, it has been reported that signaling from the type I IGF receptor uses two pathways as follows: one causes proliferation via the phosphatidylinositol 3-kinase/p70 S6 kinase pathway and the other stimulates differentiation via the mitogen-activated protein kinase pathway (10, 27, 55).

In conclusion, we have demonstrated that confluent C2C12 cells proceed actively to terminal differentiation even in mitogen-rich medium, which is clearly advantageous over mitogen-poor medium in terms of increasing differentiation. Furthermore, our data suggest that an autocrine/paracrine loop of IGFs, bFGF, and TGF-β1 selectively functions in the proliferating and differentiating cells without a depletion of serum from the cultures and that endogenous IGFBPs actively override the inhibitory effect of serum mitogens on differentiation. These results led us to consider that the generally accepted model for myogenic differentiation in vitro, which involves only the inhibitory effect of serum, may be incomplete. Therefore, we suggest that the differentiation process may proceed as long as there is an exquisite balance between mitogenic and myogenic activity of both extracellular and cellular factors.

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References


