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Transforming growth factor β induces myoblast differentiation in the presence of mitogens

(growth inhibition/myogenesis/cell cycle)

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ABSTRACT **Transforming growth factor β1 (TGF-β1) added to L_6E_9 rat skeletal myoblasts in mitogen-rich medium induces a rapid decrease in c-myc expression and delays progression through the G_1 phase of the cell cycle. This growth inhibitory response is followed by cell commitment to terminal differentiation with elevated expression of myogenin muscle determination genes, induction of muscle-specific proteins, and formation of multinucleated myotubes. These results suggest that TGF-β1 may act as a physiological inducer of myogenic differentiation in mitogen-rich environments, and its otherwise reversible growth inhibitory effect may become permanent if coupled to induction of terminal differentiation.**

The action of mitogens on cell proliferation is counterbalanced by antimitogens such as transforming growth factor β (TGF-β). By lengthening the G_1 phase in the cell cycle, TGF-β can restrict or even arrest cell proliferation (refs. 1–3; reviewed in refs. 4 and 5). Normally, the growth inhibitory effect is reversible and ends upon removal of TGF-β from the medium (1–5). Growth inhibition might become permanent, however, if slow cell cycle progression induced by TGF-β facilitated cell commitment to terminal differentiation. Often, cells that are competent to differentiate remain undifferentiated while exposed to proliferative stimuli and undergo differentiation if mitogenic stimulation passively subsides. Active interference with the proliferative stimuli of mitogens by growth inhibitors such as TGF-β might induce terminal differentiation as well.

In reality, this notion has lacked supportive evidence. Rather than promoting cell differentiation, TGF-β has been shown to inhibit differentiation of cultured myoblasts, preadipocytes, osteoblasts, and cells of other lineages (4, 6–11). In skeletal muscle myoblasts, cells whose differentiation process is understood with some detail (12–14), TGF-β interferes with the action of the muscle determination genes MyoD1 and myogenin (15–17), thereby preventing expression of muscle-specific genes, formation of myotubes, and appearance of other traits of the muscle phenotype (6–8). However, the antidifferentiative actions of TGF-β in cell culture are paradoxical given the frequent association of TGF-β with processes of tissue development and repair *in vivo*, including myogenesis. Thus, TGF-β is expressed in differentiating somites and in mesenchymal tissues during mouse embryogenesis (18–20) and during the response of myocardium to injury in experimentally induced myocardial infarction (21). TGF-β participation in cardiomyogenesis during embryo development has been established (22).

Given these two lines of contradictory evidence, the question becomes whether TGF-β is promoting differentiation *in vivo* by acting directly on myoblasts or by affecting these cells through complex indirect mechanisms such as changing their environment and whether this can be demonstrated *in vitro*. Considering this question, it is of note that myogenic differentiation has been commonly examined under conditions different from those that prevail *in vivo*. Differentiation in culture is usually induced by placing myoblasts in mitogen-poor medium (23–25), and it is myogenic differentiation triggered in this manner that is inhibited by TGF-β (6–8, 17). Mitogens retain myoblasts in a proliferative state that interferes with the ability of MyoD1 and related factors to initiate differentiation (26, 27). However, myogenic differentiation in developing tissues occurs in environments where mitogens presumably abound (28, 29), and differentiation in these tissues might depend on factors that can actively override the inhibitory effect of mitogens.

These considerations led us to test whether TGF-β could counteract the inhibitory effect of a growth-promoting environment on L_6E_9 rat skeletal myoblast differentiation. Finding this to be the case, we determined that induction of myogenic differentiation by TGF-β occurs with acute down-regulation of c-myc, a known antagonist of myogenic differentiation (27). These findings suggest that an initially reversible growth inhibitory effect of TGF-β may favor terminal myoblast differentiation with permanent withdrawal from the cell cycle.

MATERIALS AND METHODS

L_6E_9 rat skeletal muscle myoblasts (24) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). To study the effect of TGF-β, cells were plated at a density of 5×10^4 cells per cm^2 in DMEM supplemented with 20% FBS in six-well plates. Two hours after plating, the medium was changed to DMEM supplemented with 20% FBS, 10% bovine calf serum (high mitogen medium), or 5% heat-inactivated horse serum (low mitogen medium) (6, 24). Porcine TGF-β1 and -β2 were from R&D Systems (Minneapolis).

For immunostaining, cell monolayers were washed three times with phosphate-buffered saline (PBS) containing 3 mM $CaCl_2$ and 1.5 mM $MgCl_2$ (pH 7.4), fixed with 4% glutaraldehyde, and stained with a mouse monoclonal antibody against myosin heavy chain (30) followed by rhodamine-conjugated secondary antibody. Hoechst 33258 (Sigma) was used for nuclear staining.

Electrophoretic mobility-shift DNA binding assays were done with a ^{32}P-labeled 25-base-pair oligonucleotide correspondent to the myogenic factor consensus binding site from the muscle creatine kinase (MCK) enhancer region (5′-GATCCCCCCAACACCTGCTGCCTGA-3′) and using poly(dI/dC) to complete nonspecific binding as described (26, 31).

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Abbreviations: TGF-β, transforming growth factor β; FBS, fetal bovine serum; MCK, muscle creatine kinase; GAPDH, glyceraldehyde phosphate dehydrogenase.

For MCK assays, cell monolayers were washed twice with PBS, lysed with 0.25 ml of 50 mM glycylglycine buffer (pH 5.5), and stored at −20°C until assayed. After thawing, MCK activity was assayed with a spectrophotometric assay kit (Sigma). Total soluble protein in the samples was assayed with Bradford reagent (Bio-Rad).

For Northern blot mRNA assays, total RNA was isolated and, where indicated, enriched for poly(A)$^+$ RNA by chromatography over oligo(dT)-Sepharose as described (32). RNA was separated on 1% agarose gels containing formaldehyde and transferred to Duralon-UV membranes (Stratagene). Specific mRNAs were detected using as probes cDNAs corresponding to human *MYC* (pcmycc41-3R) (33) or myogenin (pUC65-2) (34) labeled with ^{32}P by random priming (Multiprime; Amersham). As a control, the filters were probed with ^{32}P-labeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (pRGAPDH-13) (35). Hybridization signals were recorded, digitized, and linearly quantified using a Phosphorimager 400-E and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Background values were determined from equivalent surface areas near each hybridization signal and were subtracted from each value. The specific c-myc and myogenin mRNA signal values were normalized relative to the GAPDH signal in the same lane.

For flow cytometry, cells were detached with 0.25% trypsin/1 mM EDTA in PBS, washed with PBS, fixed with 70% ethanol in PBS, treated with RNase A, and mixed with propidium iodide (50 μg/ml). Stained cells were analyzed in a FACScan flow cytometer with data processing Lysis II software (Becton Dickinson).

For growth inhibition assays, cells were incubated with ^{125}I-labeled deoxyuridine (^{125}I-dU) for 2 h, washed with ice-cold PBS containing 3 mM $CaCl_2$ and 1.5 mM $MgCl_2$ (pH 7.4), and fixed for 1 h with 95% methanol on ice. After two washes with PBS, cells were lysed for 8 h with 0.5 ml of 0.2 M NaOH, and radioactivity in the cell lysates was counted.

RESULTS

TGF-β Induces Myogenic Differentiation. L_6E_9 rat skeletal muscle myoblasts can differentiate when placed in mitogen-poor medium (24). If maintained with serum mitogens, L_6E_9 cells grow forming a confluent, contact-inhibited monolayer that remains largely undifferentiated for several days (ref. 24; Fig. 1*a*). However, L_6E_9 cultures differentiated if they received TGF-β as they were reaching confluence in mitogen-rich medium. Differentiation was evident by the formation of numerous myotubes (Fig. 1*b*) that were multinucleated (Fig. 1*c*) and expressed myosin heavy chain (Fig. 1*d*). L_6E_9 cell differentiation was detectable with 5 pM TGF-β1 and was maximal with TGF-β1 concentrations 50 pM or higher (data not shown). Myotubes began to appear ≈72 h after TGF-β addition and the response was maximal 72 h later. Similar effects were observed with the isoform TGF-β2.

The response of L_6E_9 cells to TGF-β1 was a typical myogenic differentiation process according to various early markers of cell commitment to differentiation. Thus, differentiation in response to TGF-β1 occurred with a marked up-regulation of the steady-state level of myogenin mRNA (Fig. 2*A*). Myogenin is one of the members of the HLH family of transcription factors that can activate muscle-specific gene expression initiating myogenic differentiation (12, 34, 36, 37). Gel-retardation DNA binding assays using an oligonucleotide corresponding to the myogenin/MyoD1 binding site from the enhancer of the MCK gene (17, 26, 31, 37) were carried out to determine the presence of enhancer-binding proteins in L_6E_9 cells. This oligonucleotide formed two specific complexes with L_6E_9 nuclear proteins (Fig. 2*B*). The specificity of these complexes was demonstrated by their resistance to

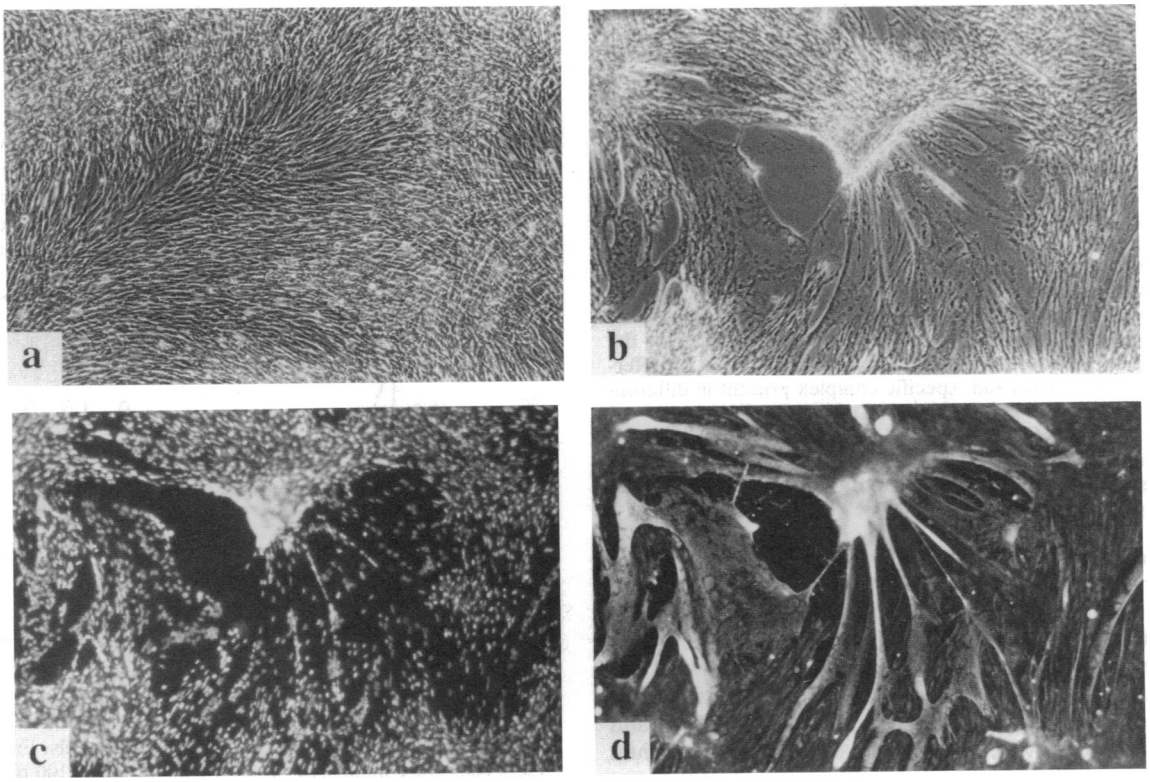

FIG. 1. TGF-β induces L_6E_9 myoblast differentiation. Cells (5×10^4 cells per cm^2) freshly seeded in medium containing 20% FBS received no additions (*a*) or 50 pM TGF-β1 (*b–d*). Six days after plating, micrographs were obtained of the cell monolayers (*a* and *b*), fluorescently labeled nuclei (*c*), or indirect myosin heavy-chain immunofluorescence (*d*). (*b–d*) The same field. The clump of cells in this field is typical of the TGF-β response in this cell line. (×135.)

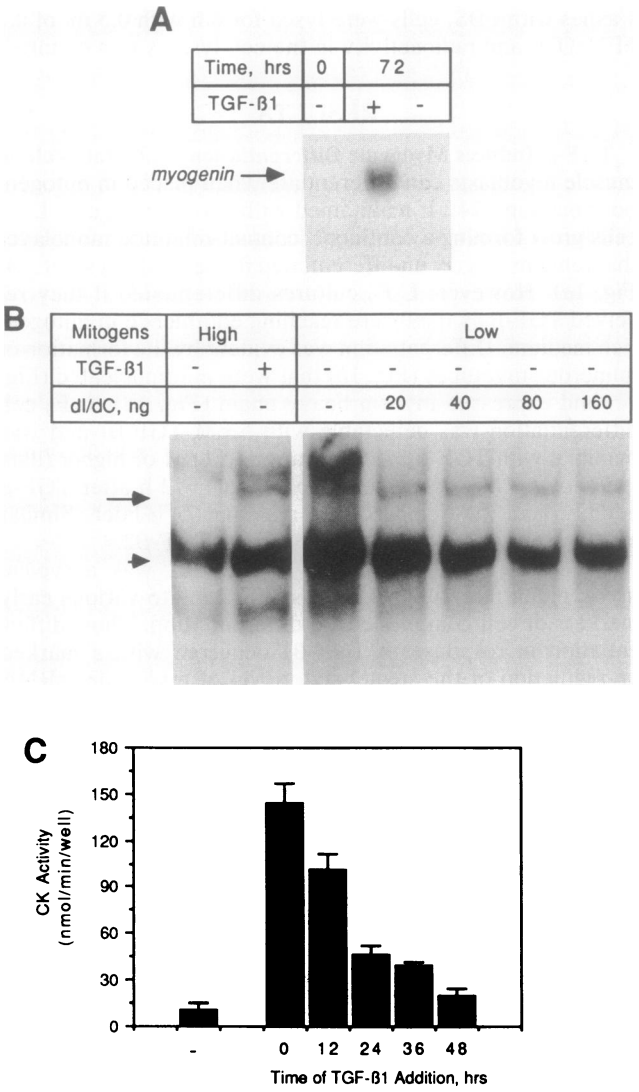

FIG. 2. TGF-β induces myogenic differentiation markers. (*A*) Myogenin mRNA detected by Northern blot analysis of total RNA (20 μg per lane) isolated at the time of plating (0 h) or after a 72-h incubation in the presence (+) or absence (−) of 50 pM TGF-β1. (*B*) Gel-shift assay using ^{32}P-labeled MCK enhancer oligonucleotide mixed with extracts from cells cultured for 72 h in high-mitogen (20% FBS) or low-mitogen (5% heat-inactivated horse serum) medium with (+) or without (−) TGF-β1 (100 pM). The indicated amounts of poly(dI/dC) were present during incubation of extracts with the specific oligonucleotide. Arrow, specific complex present in differentiating cultures; arrowhead, specific complex present in differentiating as well as proliferating cultures. (*C*) Control cells (−) received no TGF-β1 and the rest received 50 pM TGF-β1 at the time of plating (0 h) or at later time points as indicated. Cell extracts prepared 72 h after plating were assayed for MCK activity. Results are the average ± SD of triplicate samples.

an excess of the control oligonucleotide poly(dC/dI). One of these complexes was observed with extracts from both differentiated and undifferentiated cells (Fig. 2*B*) and might be related to a similar complex previously observed in nonmuscle cells (37). The other specific complex was observed only with extracts from differentiated cultures and was similar in cells differentiated by the action of TGF-β1 and cells differentiated by being placed in mitogen-poor medium (Fig. 2*B*).

Enzyme activity assays confirmed a marked induction of MCK in TGF-β1-treated L_6E_9 cells relative to controls maintained in the presence of mitogen-rich medium alone (Fig. 2*C*). This assay and visual inspection of the monolayers were also used to determine the effect of cell density on the response to TGF-β1. The differentiative response was maximal when TGF-β1 was added to freshly plated subconfluent (5×10^4 cells per cm^2) monolayers and became less intense when TGF-β1 was added as cells became confluent with time in culture (Fig. 2*C*).

Rapid Loss of c-myc Expression and Accumulation of Cells in G_1 Phase Precede Differentiation. Proliferating myoblasts contain basal levels of myogenin, MyoD1, and/or related myogenic differentiation factors, but countervailing factors prevent initiation of the differentiation process (14, 25, 34, 36–39). c-myc, which promotes progression through the G_1 phase of the cell cycle (40–42), is one of the negative regulators of myogenic differentiation induced by myogenin or MyoD1 (27, 43, 44). The levels of c-myc are high in proliferating myoblasts and decline after removal of mitogens from the medium, allowing differentiation to occur (45).

Because TGF-β1 can down-regulate c-myc expression in various cell types (4, 5, 42, 46), thus accounting, at least partially, for the growth inhibitory activity of this factor (42), we examined the effect of TGF-β1 on c-myc expression in proliferating L_6E_9 cells. Northern blot assays showed a marked (>90%), rapid, and persistent down-regulation of c-myc mRNA levels in response to TGF-β1 (Fig. 3). This effect was nearly maximal 1.5 h after TGF-β1 addition to cells and persisted for at least 6 h. Thus, TGF-β action rapidly removed this antagonist of myogenic differentiation.

The down-regulation of c-myc expression by TGF-β1 was associated with an accumulation of cells in G_1 phase as determined by flow cytometry (Fig. 4*A*). This resulted in a net growth inhibitory effect that was detected as a decrease in the rate of ^{125}I-dU incorporation into DNA (Fig. 4*B*), similar to the previously noted inhibition of DNA synthesis by TGF-β1 in L_6 cells (47).

DISCUSSION

Proliferation and terminal differentiation appear to be mutually exclusive in myoblasts. We tested whether an inhibitor of cell proliferation, TGF-β, might concomitantly act as an inducer of terminal differentiation with permanent cell withdrawal from the proliferative cycle. Our results show that two TGF-β isoforms, β1 and β2, can override the antimyogenic action of serum mitogens on L_6E_9 rat skeletal myoblasts and induce terminal differentiation. Added to L_6E_9 cells in the presence of mitogens, TGF-β induces the appearance of

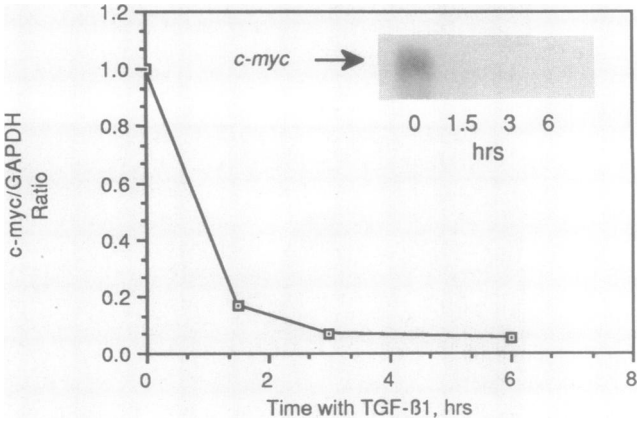

FIG. 3. Effect of TGF-β1 on c-myc mRNA levels. Freshly plated L_6E_9 cells were incubated for 24 h with TGF-β1 (100 pM) added at the indicated times before the end of the incubation. Poly(A)$^+$ RNA from these cells was subjected to c-myc mRNA Northern blot assay (*Inset*). After removal of the c-myc probe, the blot was hybridized with a GAPDH probe. Digitized c-myc and GAPDH hybridization signals were used to obtain the normalized c-myc mRNA values.

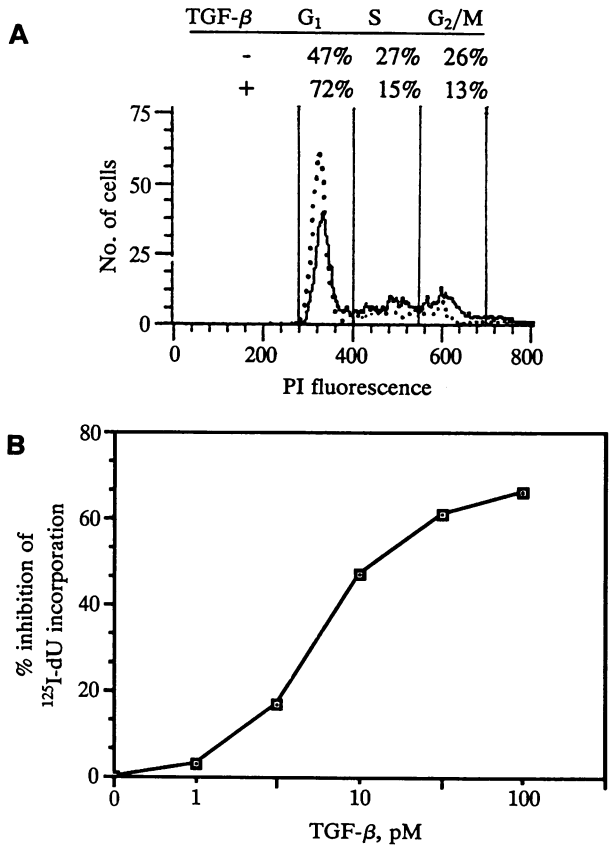

FIG. 4. Growth suppressive effect of TGF-β1 on L_6E_9 cells. Cells freshly seeded in medium containing 20% FBS received 50 pM TGF-β1 (+) or no additions (−). (*A*) Forty-eight hours after plating, cells were subjected to flow cytometry analysis. Cell cycle distributions of untreated control cells (solid trace) and TGF-β-treated cells (dotted trace) were integrated and are expressed as percentage of the total cell population (*Upper*). (*B*) Inhibition of ^{125}I-dU incorporation into DNA 48 h after addition of increasing concentrations of TGF-β1. PI, propidium iodide.

myotubes with traits typical of an overtly differentiated muscle phenotype. The biochemical and morphological properties of TGF-β-induced myotubes are similar to those of myotubes spontaneously arising in cultures placed in mitogen-poor medium (6, 24). Furthermore, increased expression of the muscle determination gene myogenin and appearance of proteins that bind to the muscle-specific MCK gene enhancer region are early events during TGF-β-induced L_6E_9 myoblast differentiation as they are in differentiation induced by placing myoblasts in mitogen-poor medium (37).

Induction of L_6E_9 myoblast differentiation by TGF-β is preceded by a growth inhibitory response. This response is characterized by a rapid and persistent decline in c-myc expression and an accumulation of cells in G_1 phase. A decline in c-myc expression induced by TGF-β could interfere with G_1/S-phase transition in the proliferative cycle (5, 42). The decrease in c-myc expression is, therefore, a sign if not a cause of an altered cell cycle progression in TGF-β-treated L_6E_9 cells.

When myoblasts are induced to differentiate by being placed in mitogen-poor medium, they withdraw from the proliferative cycle during G_1 phase (24, 25). Since TGF-β lengthens G_1 phase in L_6E_9 cells, it is possible that their differentiation is the result of an impaired G_1 phase progression. An attractive possibility is that the molecular events that lengthen G_1 phase in response to TGF-β might also be specifically involved in triggering terminal differentiation. Indeed, c-myc appears to interfere with the function of muscle determination factors MyoD1 and myogenin through direct or indirect interactions with these nuclear proteins (27, 43, 44). This interference would be alleviated when the levels of c-myc decrease in response to TGF-β.

Contact-inhibited L_6E_9 cells did not differentiate even with TGF-β addition. Therefore, any role for reduced c-myc levels in triggering L_6E_9 differentiation would seem to depend on additional events taking place in cycling cells. The nature of these events is unknown to us. The growth suppressor gene product pRB is one potential target of TGF-β action during G_1 phase (3, 5). However, the response to TGF-β1 in L_6E_9 cells included a marked decrease in total pRB protein level (A.Z., unpublished work), an effect that defies any simple model for a role of pRB in TGF-β-induced myoblast differentiation. The specific interactions of pRB with other nuclear factors (48–52) and their functional significance need to be elucidated in order to determine their role, if any, in the differentiative response to TGF-β.

The myogenic effect of TGF-β observed in the present studies relates to the proposed involvement of this factor in myogenic development and repair processes *in vivo* (18, 20–22). TGF-β might act as an inducer of differentiation in mitogen-rich environments *in vivo* as it does in L_6E_9 cell cultures. These observations contrast, however, with the previously observed inhibitory effect of TGF-β on myogenic differentiation in mitogen-poor medium *in vitro*. With the exception of adult rat cardiomyoblasts (53), myoblast differentiation in mitogen-poor medium is inhibited by TGF-β (6–8, 15, 16). The mechanism of this effect is different from the mechanism by which serum mitogens inhibit differentiation (17). The antimyogenic effect of TGF-β in a mitogen-poor environment could reflect the existence of a physiological process to prevent premature myoblast differentiation before sufficient tissue mass has accumulated.

The response of L_6E_9 cells to TGF-β is affected by cell density and cell interaction with the extracellular matrix (16) in addition to the hormonal environment. The influence of these variables on TGF-β responsiveness might be cell specific. We note that two other myoblast cell lines, C_2C_{12} and P_2, did not differentiate when placed in high serum and TGF-β1 conditions similar to those that promote L_6E_9 differentiation (A.Z., unpublished work). It has been emphasized (54) that growth factors are multifunctional and that the responses that each of them incites depend as much on the nature and environment of the target cells as they do on the factors themselves.

Note Added in Proof. We have recently observed that the mRNA levels of another inhibitor of differentiation, Id (26), decline rapidly ($t_{1/2}$, 2 hr) after addition of TGF-β to L_6E_9 cells. Thus, TGHF-β action rapidly removes two antagonists of myogenic differentiation, c-myc and Id,in L_6E_9 myoblasts.

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