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Type β transforming growth factor is an inhibitor of myogenic differentiation

(myogenesis-inhibiting factor/muscle development/platelets/collagen/growth factor receptors)

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ABSTRACT **We have investigated the effect of type β transforming growth factor (TGF-β) on the differentiation of skeletal muscle myoblasts. TGF-β potently ($ID_{50} \approx 10$ pM) prevents established cell lines and primary cultures of rat and chicken embryo myoblasts from fusing into multinucleated myotubes. Inhibition of morphological differentiation by TGF-β correlates with inhibition of the expression of muscle-specific mRNAs and proteins, strong induction of extracellular matrix type I collagen and fibronectin, and a marked tendency of the treated myoblasts to aggregate into densely multilayered arrays or clusters. Myogenic differentiation can resume after removal of TGF-β from the medium. Examination of the time of action of TGF-β shows that myoblasts stochastically reach a point beyond which they become insensitive to the inhibitory action of TGF-β. This resistance of committed myoblasts to the inhibitory action of TGF-β is not associated with any measurable change in the number or affinity of TGF-β receptors in those cells. The results indicate that TGF-β is a potent inhibitor of myogenesis and may regulate muscle development *in vivo*.**

Transforming growth factor β (TGF-β), a hormonally active polypeptide found in normal and transformed tissues, is a potent regulator of cell development (for review, see ref. 1). At picomolar concentrations TGF-β induces anchorage-independent growth of fibroblasts but inhibits the growth of certain tumor-derived as well as normal cells (2–6). In addition to its effects on cell proliferation, TGF-β inhibits adipogenic differentiation without altering the growth rate of preadipocytes (7). Many cells, the growth or differentiation of which is regulated by TGF-β, respond to this factor with a marked increase in the production and accumulation of the extracellular matrix proteins fibronectin (8) and collagen (8, 9). Available evidence suggests that the induction of an abundant extracellular matrix by TGF-β mediates cellular responses to this factor such as the stimulation of anchorage-independent proliferation (8). These actions of TGF-β are presumably mediated by specific cell surface receptors: three structurally distinct cell surface glycoproteins that exhibit the properties of high-affinity receptors for TGF-β have been identified in mammalian and avian cells (10–12). It is not known whether all three receptor forms are involved in the mediation of TGF-β actions or whether one receptor form is a signaling receptor, whereas the others have some other function(s).

The widespread distribution of TGF-β and its receptors in different cell types and tissues suggests that this factor is involved in an ample spectrum of developmental processes *in vivo*. To obtain further information on the range of cellular targets for TGF-β, we have investigated the effects of this factor on the differentiation of skeletal muscle myoblasts. These studies reveal a strong inhibitory action of TGF-β on myogenesis.

METHODS

L_6E_9 rat skeletal muscle myoblasts (13) were grown in Dulbecco's modified Eagle (DME) medium supplemented with 20% fetal calf serum (growth medium). For differentiation subconfluent L_6E_9 cells were shifted to DME medium containing 7% heat-inactivated horse serum (differentiation medium). L_8 rat muscle cells (ref. 14; obtained from R. Singer, University of Massachusetts) were grown in DME medium supplemented with 10% heat-inactivated horse serum and allowed to spontaneously differentiate after reaching confluency in the same medium. Chicken myoblasts prepared from collagenase-digestion of minces of day 12 embryos (provided by R. Singer) were plated with minimal essential medium supplemented with 10% fetal calf serum plus 5% chicken serum, conditions under which they spontaneously differentiate.

The purification of TGF-β from human platelets and preparation of ^{125}I-labeled TGF-β (275 Ci/g; 1 Ci = 37 GBq) have been described (10). Isolation of cytoplasmic L_6E_9 cell RNA and RNA blot analysis of these RNA preparations with a pool of nick-translated ^{32}P-labeled cDNA plasmids specific for the indicated mRNAs were performed as described (15). For one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) (16) of [^{35}S]methionine-labeled cellular proteins, cells were lysed by heating in sample buffer (16) containing DNase I (1 mg/ml) and 2 mM diisopropyl fluorophosphate. For two-dimensional isoelectric focusing $NaDodSO_4$/PAGE, cells were lysed in isoelectric focusing lysis buffer (17) containing 2 mM diisopropyl fluorophosphate, and the supernatant was loaded onto the gel after centrifugation. Isolation and analysis of ^{35}S-labeled extracellular matrix collagen (8), DNA determinations (18), and TGF-β receptor affinity labeling in intact cell layers (10, 11) were done as previously described. For determinations of creatine phosphokinase activity, cells were lysed in the presence of 50 mM glycylglycine buffer, pH 7.0, and the enzymatic activity in the soluble extracts was measured using a commercially available kit (Sigma). Further details of specific experiments are given in the corresponding figure legends.

RESULTS

TGF-β Prevents Spontaneous Myogenic Fusion. Placed in culture conditions that favor myogenic differentiation, L_6E_9 and L_8 rat skeletal muscle myoblasts, as well as primary chicken embryo myoblasts, progressively expressed the differentiated morphology characterized by extensive fusion

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Abbreviations: TGF-β, type β transforming growth factor; CPK, creatine phosphokinase.

of cells into multinucleated myotubes (Fig. 1 *a*, *c*, and *e*). The addition of human platelet-derived TGF-β to the medium completely prevented the morphological differentiation of all three types of myoblasts (Fig. 1 *b, d*, and *f*). In the presence of TGF-β the cells remained unfused and mononucleated, and the established rat myoblast lines had a marked tendency to aggregate as densely multilayered arrays or clusters of cells (Fig. 1 *b* and *d*). TGF-β had little or no effect on the cell cycle of myoblasts as demonstrated by the fact that the rate of DNA accumulation was not altered by TGF-β in L_6E_9 cells that had been shifted to differentiation medium and was only slightly decreased by TGF-β in exponentially growing cells (data not shown). Cells treated with TGF-β remained unfused as long as the medium was replenished with TGF-β regularly. However, the effect of TGF-β was reversible, as replating the cells in differentiation medium lacking TGF-β allowed normal expression of the differentiated phenotype (data not shown).

Biochemical Differentiation Is also Inhibited by TGF-β. Myogenic differentiation is characterized by a marked increase in the expression of genes encoding muscle-specific proteins, including myosin heavy and light chains, α-actin and troponin T (19–25). The levels of mRNAs that correspond to these proteins in differentiating L_6E_9 myoblasts progressively increase, a change that is prevented by TGF-β (Fig. 2). Furthermore β- and γ-actin mRNA levels, which are high in undifferentiated myoblasts and decrease sharply during differentiation, remained high in the TGF-β-treated cells as shown by the same figure. However, differentiation-related changes in specific mRNAs began to be noticeable 6–9 days after the addition of TGF-β if the medium had not been periodically replenished with this factor (data not shown), a phenomenon probably due to the depletion of TGF-β in the medium. The reversibility of the inhibitory action of TGF-β on myogenesis was further documented with the expression of differentiation markers in cells rescued from the inhibited state by being transferred to TGF-β-free differentiation medium (Fig. 2).

L_6E_9 cells in which differentiation had been inhibited by TGF-β did not display muscle-specific proteins when extracts from ^{35}S-labeled cultures were displayed in one- and two-dimensional gels (Fig. 3). The only exception is a low level of expression of α-tropomyosin in TGF-β-treated cells (Fig. 3), which is similar to the level detected in myoblasts growing at an exponential rate (data not shown). In contrast, the production of proteins of about 240 kDa, 190 kDa, and 170 kDa was markedly elevated in TGF-β-treated cells (Fig. 3). These proteins are tentatively identified as fibronectin, procollagen α_1(I), and procollagen α_2(I) based on their molecular properties, the known presence of fibronectin and type I collagen in L_6 cells (27), and the known ability of TGF-β to increase markedly the production of extracellular matrix type I collagen and fibronectin in many cell lines including skeletal muscle myoblasts (8). The experiment of Fig. 4 corroborates this conclusion and demonstrates the potency of TGF-β in elevating the incorporation of procollagens α_1(I) and α_2(I) into the extracellular matrix of L_6E_9 cells.

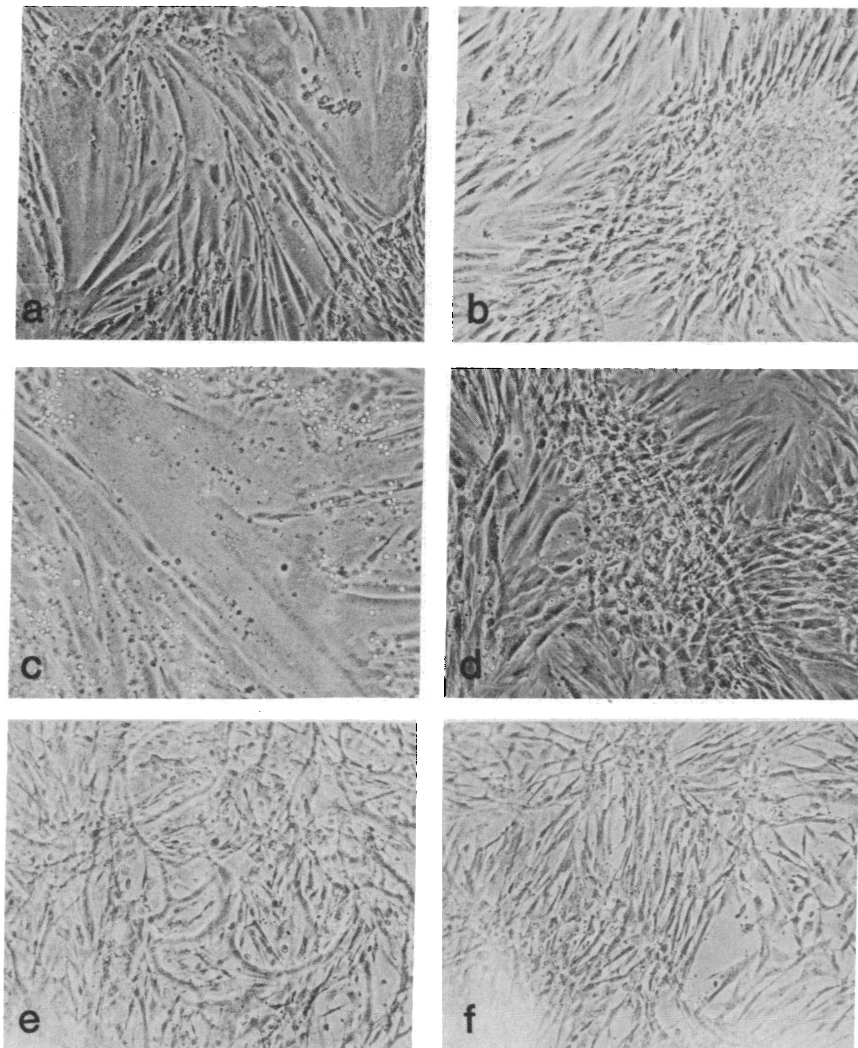

FIG. 1. Inhibition of morphological differentiation by TGF-β. L_6E_9 rat myoblasts (*a*, *b*) L_8 rat myoblasts (*c*, *d*) and primary chicken embryo myoblasts (*e*, *f*) at conditions conducive to myogenic differentiation without (*a*, *c*, *e*) or with 200 pM TGF-β (*b*, *d*, *f*). Photographs were taken 6 days (*a–d*) or 24 hr (*e*, *f*) later. Note extensive formation of myotubes in the untreated controls, the inhibition of this process by TGF-β, and the large arrays of clustered cells in the TGF-β-treated rat myoblasts.

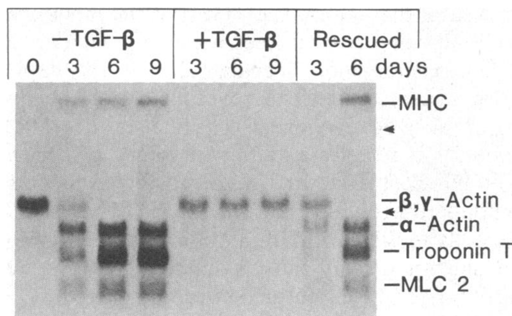

FIG. 2. Inhibition of biochemical differentiation by TGF-β. L_6E_9 cells cultured in growth medium were shifted into differentiation medium with (+TGF-β) or without (−TGF-β) 0.3 nM TGF-β on day 0. Cells receiving TGF-β were replenished with this factor at 0.3 nM every 3 days. Cytoplasmic RNA was obtained from these cultures on days 3, 6, or 9. Other cultures were kept in the presence of 0.3 nM TGF-β for 3 days and then transferred (*Rescued*) back to differentiation medium lacking TGF-β for another 3–6 days before RNA isolation. RNA (10 μg) from each experimental condition was subjected to RNA transfer hybridization analysis with a pool of ^{32}P-labeled cDNA plasmids corresponding to the indicated proteins (15). Shown is a resulting autoradiogram. MHC, myosin heavy chain; MLC 2, myosin light chain 2. Arrowheads mark the positions corresponding to the 18S and 28S rRNAs.

Resistance to TGF-β After Cell Commitment to Differentiation. Creatine phosphokinase (CPK) is another marker of myogenic differentiation the activity of which was inhibited in TGF-β-treated myoblasts. CPK activity was used to determine in detail the parameters of TGF-β action on L_6E_9 cells. The degree of inhibition of CPK expression induced by TGF-β decreased progressively in cells that had been exposed for lengthening periods of time to conditions conducive to differentiation before TGF-β was added (Fig. 5). In these experiments, TGF-β proved completely ineffective when

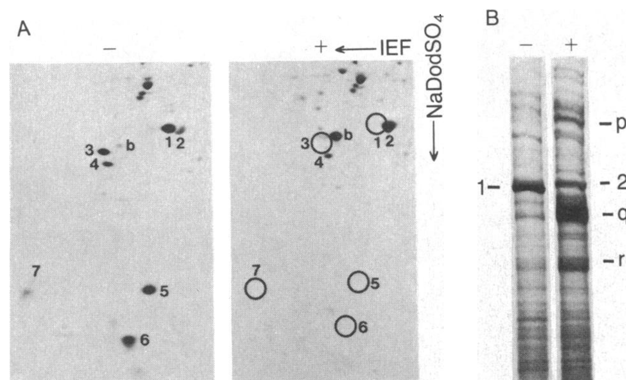

FIG. 3. Two-dimensional analysis of cellular proteins. L_6E_9 cells maintained for 6 days in differentiation medium with (+) or without (−) TGF-β (0.3 nM, replenished every 3 days) were labeled by incubation for 4 hr in the presence of [^{35}S]methionine (100 μCi/ml). Cells were then rinsed with cold 20 mM sodium phosphate at pH 7.4, 150 mM NaCl, lysed, and subjected to isoelectric focusing followed by $NaDodSO_4$/PAGE in the second dimension (*A*) or directly subjected to one-dimensional $NaDodSO_4$/PAGE (*B*). Only relevant portions of the autoradiograms from the gels are shown. The proteins identified in *A* (according to ref. 26) are as follows: 1, α-actin; 2, β- and γ-actin; 3, β-tropomyosin; 4, α-tropomyosin; 5, embryonic myosin light chain 1; 6, myosin light chain 2; 7, troponin C; b, unidentified protein induced by TGF-β. Proteins 1 and 3–7 are induced during differentiation. α-Tropomyosin is present in myoblasts, and the level is elevated in myotubes. The proteins identified in *B*: 1, muscle-specific embryonic myosin heavy chain; 2, nonmuscle myosin heavy chain; p, q, r, proteins of 240 kDa, 190 kDa, and 170 kDa, respectively, induced by TGF-β.

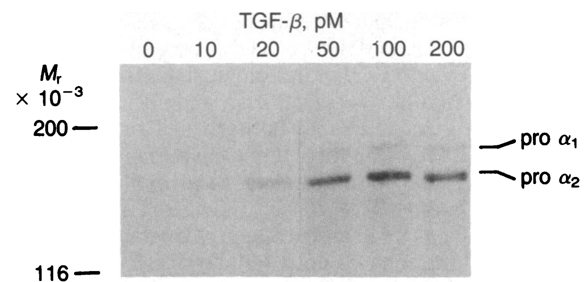

FIG. 4. Effect of TGF-β on extracellular matrix procollagen levels. Subconfluent L_6E_9 cells received the indicated concentrations of TGF-β. Sixteen hours later, the cultures were transferred to cysteine-free minimal essential medium containing 20 μCi of [^{35}S]cysteine per ml and the corresponding concentration of TGF-β. After 3 hr of labeling, the monolayers were rinsed and extracted with urea-containing buffer to release extracellular matrix proteins (8). The urea extracts were displayed by $NaDodSO_4$/PAGE and fluorography to visualize procollagens α_1(I) and α_2(I) (8). The positions corresponding to molecular weight markers of 200,000 and 116,000 are indicated.

added to the cultures 3 days after their shift to differentiation medium. This phenomenon is consistent with the stochastic mode of L_6E_9 cell commitment to terminal differentiation (13) and demonstrates a critical point after which L_6E_9 cells become resistant to the inhibitory action of TGF-β on myogenesis. However, differentiated L_6E_9 cultures can still respond to TGF-β with increased expression of collagen and fibronectin (data not shown).

Relationship Between Saturation of TGF-β Action and TGF-β Receptors. Fig. 6 shows the dose–response relationship for the action of TGF-β on CPK activity. Enzyme assays performed on cells 3 days after their shift to differentiation medium showed that inhibition of CPK expression by TGF-β was half maximal and saturated in cells that had received 10 pM and 50 pM hormone, respectively, on the day of the shift. If assays were performed when full differentiation of the untreated controls had already occurred, the inhibitory action of TGF-β was half maximal and saturated in wells that had received 50 pM and 100 pM TGF-β, respectively. This change in the dose–response relationship with time is attrib-

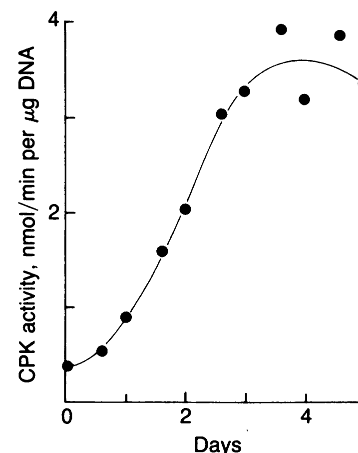

FIG. 5. Time of action of TGF-β. L_6E_9 cells were transferred to differentiation medium, and at the indicated times after the transfer cultures received 0.2 nM TGF-β. Six days after the initial shift to differentiation medium, cells in all experiments were scraped into 50 mM glycylglycine buffer at pH 7.0 and homogenized, and the soluble extracts were subjected to CPK activity assay and DNA determination. CPK activity is expressed as nmol of NADH produced per min under standard reaction conditions and is normalized to the DNA content of each well. The DNA content was not significantly altered by TGF-β. Data are the average of duplicate determinations.

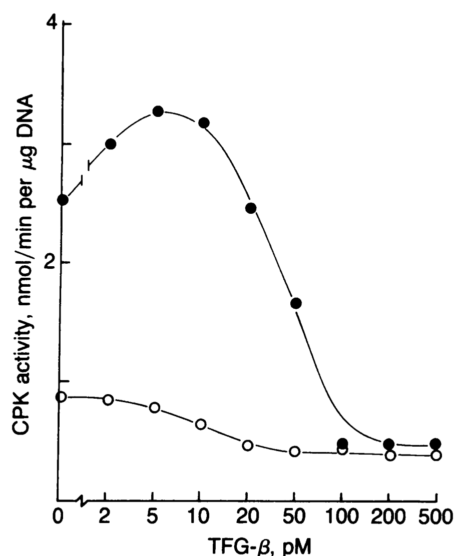

FIG. 6. Concentration-dependence of TGF-β effect on CPK. L_6E_9 cells were shifted to differentiation medium containing the indicated concentrations of TGF-β. CPK activity in the cultures was determined 3 (○) or 6 (●) days after the media shift and is expressed as in Fig. 5.

utable to degradation of TGF-β after prolonged incubation with the cells. Control experiments showed that 3 and 6 days after one single addition of 50 pM ^{125}I-labeled TGF-β to L_6E_9 cells, 41% and 53% of the added radioactivity, respectively, had been converted into tricholoroacetic acid-soluble material.

Characterization of TGF-β receptors by affinity-labeling of L_6 and L_8 myoblasts with ^{125}I-labeled TGF-β had previously demonstrated the presence of two structurally distinct receptor types termed respectively, the type I (65 kDa) and type II (85 kDa) TGF-β receptors (12). Fig. 7 illustrates the presence of both types of receptors in L_6E_9 myoblasts as well as in extensively fused myotubes. Unlabeled TGF-β competed 2- to 5-fold more effectively for binding to type I receptors than to type II receptors in both sets of cells as determined by densitometry (data not shown) of the autoradiogram shown in Fig. 7. Thus, the binding-competition curve for type I receptors (apparent $K_d \approx$ 30pM TGF-β) approached most closely the dose–response relationship of TGF-β action on CPK. No major differences were observed in receptor content or affinity between dishes of confluent myoblasts and fully differentiated myotubes.

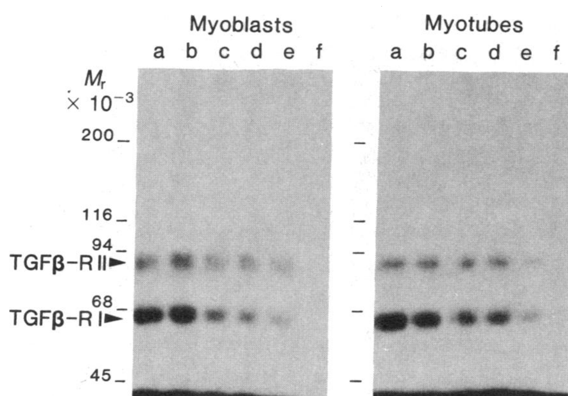

FIG. 7. TGF-β receptors affinity labeled in L_6E_9 myoblasts and myotubes. Subconfluent L_6E_9 myoblasts and fully differentiated (>90% cell fusion) myotubes were incubated for 3.5 hr at 4°C with binding buffer containing 20 pM ^{125}I-labeled TGF-β without (lanes a) or with 20 pM (lanes b), 40 pM (lanes c), 80 pM (lanes d), 200 pM (lanes e), or 500 pM (lanes f) unlabeled TGF-β. Cells were then crosslinked with cell-bound radioactivity using 0.2 mM disuccinimidyl suberate and extracted with buffer containing Triton X-100 and protease inhibitors (10, 11). The extracts were displayed by $NaDodSO_4$/PAGE and autoradiography (3 days). The autoradiographic bands corresponding to affinity-labeled type I (TGFβ-RI) and type II (TGFβ-RII) TGF-β receptors are indicated. Numbers on the left refer to the position and molecular weight of the protein standards.

DISCUSSION

The results reported here show that TGF-β strongly inhibits the terminal differentiation of mammalian and avian skeletal muscle myoblasts as shown by morphological and biochemical criteria. The inhibitory action of TGF-β is not restricted to established myoblast cell lines, as TGF-β readily inhibits the fusion of primary cultures of chicken embryo myoblasts. The inhibitory effect of TGF-β is reversible and does not result from selective induction of proliferation of a nondifferentiating subpopulation of cells. The results also indicate that TGF-β cannot inhibit myogenic differentiation and continued muscle-specific gene expression once cell commitment to differentiation has occurred. The proliferation of exponentially growing myoblasts was unaffected or only slightly inhibited by TGF-β (data not shown). This apparent lack of intrinsic mitogenic activity is consistent with the action of TGF-β on other cell types of mesenchymal origin (5, 6). The inhibitory effect of TGF-β on myogenesis is therefore unusual and differs from the effects of serum mitogens and fibroblast growth factor that tend to delay myoblast fusion by retaining the cells in a proliferative state (13, 28). TGF-β is a chemically defined polypeptide that inhibits myogenesis without inducing cell proliferation, but it now seems likely that TGF-β is the molecular entity responsible for the antimyogenic activity previously found in medium conditioned by a cell line derived from newborn rat liver BRL-3A cells (29); BRL-3A cells secrete TGF-β (30).

The induction of a rapid increase in fibronectin and/or collagen levels by TGF-β in many cell types, the ability of exogenous fibronectin to substitute for TGF-β in the induction of anchorage-independent growth of fibroblasts, and the inhibition of TGF-β-induced anchorage-independent cell proliferation by competitive inhibitors of fibronectin binding have led to the proposal that TGF-β effects at the extracellular matrix level may mediate cellular responses to this factor (8). Manipulation of the extracellular matrix composition by addition of exogenous fibronectin and/or collagen has been reported to inhibit myogenic fusion of L_6 myoblasts (31) and adipogenic differentiation of 3T3 mouse preadipocytes (32). That inhibition of both differentiation processes by TGF-β correlates with the ability of TGF-β to markedly increase collagen and fibronectin production by myoblasts and predipocytes (this report and refs. 7 and 8) is noteworthy. Furthermore, the morphology of L_6 myoblasts in which exogenous fibronectin had altered the extracellular matrix composition was remarkably similar to the morphology of TGF-β-treated myoblasts observed in these studies: both cell types included the formation of arrays of densely clustered mononuclear cells (31). These observations support the hypothesis that the effects of this factor at the extracellular matrix level mediate the control of myogenic differentiation by TGF-β.

Crosslinking of rat skeletal muscle myoblasts and myotubes with cellbound ^{125}I-labeled TGF-β reveals the presence of 65-kDa (type I) and 85-kDa (type II) affinity-labeled TGF-β receptor forms. As noted before in L_6 and L_8 myoblasts (11), L_6E_9 cells do not exhibit the 560-kDa disulfide-linked receptor form that predominates in many other cell types (9–11). The dose–response relationship of the inhibitory action of

TGF-β on myogenesis parallels more closely the ligand saturation curve of type I TGF-β receptors than of type II TGF-β receptors, raising the possibility that the former receptor type mediates this action of TGF-β. However, the possibility that fractional occupancy of type II receptors by TGF-β is sufficient to mediate a full inhibitory effect through this receptor type cannot be dismissed. The results also indicate that the resistance to the inhibitory action of TGF-β in L_6E_9 cells committed to terminal differentiation does not occur from a loss of cell surface receptors for TGF-β. Furthermore, the ability of differentiated L_6E_9 cell cultures to respond to TGF-β with increased production of collagen and fibronectin demonstrates that the TGF-β receptors in these cells remain functionally active after differentiation.

These studies identify TGF-β as a potent modulator of skeletal muscle development, at least *in vitro*. TGF-β is widely distributed among tissues, is abundant in platelets (33), and may be secreted by activated T-lymphocytes involved in the inflammatory response (9). Therefore, TGF-β is likely to be readily available and actively involved in processes of myogenic development that occur during embryogenesis and after muscle injury. Although the consequences of potential synergistic interactions between TGF-β and other factors during embryogenesis and regenerative processes are not known, the present results suggest that TGF-β might act *in vivo* to delay terminal differentiation of myoblasts or muscle satellite cells until formation of an adequate connective tissue and vasculature has occurred.

Note Added in Proof. Hybridization of the filters shown in Fig. 2 with ^{32}P-labeled cDNA plasmids corresponding to fibronectin and $\alpha 2(I)$ collagen showed a sharp increase in the corresponding mRNAs in cells treated with TGF-β relative to untreated myoblasts or myocytes.

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