**Communication**

**Cell Adhesion to Collagen and Decreased Myogenic Gene Expression Implicated in the Control of Myogenesis by Transforming Growth Factor β**

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Transforming growth factor β (TGF-β1) is an inhibitor of skeletal muscle myoblast differentiation. Myoblast differentiation is dependent on the expression of certain myogenic differentiation genes and is affected by cell interaction with the extracellular matrix. We have searched for events in the differentiation process of L929 rat myoblasts that may be involved in the inhibitory action of TGF-β1. Elevated expression of the myogenic differentiation gene, myogenin, which is thought to play a central role in the differentiation process, occurs 10 h after the shift of L929 myoblasts to differentiation medium. Elevation of myogenin mRNA is blocked by TGF-β1 added at the time of the shift. This effect is preceded by, and might be related to, a rapid up-regulation of junB mRNA observed in TGF-β1-treated L929 myoblasts. However, TGF-β1 can also block myogenic differentiation in cells transfected with the myogenin gene under the control of a constitutive SV40 viral promoter. The nature of a mechanism that could mediate the inhibitory action of TGF-β1 without blocking myogenin mRNA expression is suggested by the observations that (a) TGF-β1 up-regulates type I collagen expression and deposition in L929 myoblasts, (b) a fibrillar type I collagen layer inhibits L929 myoblast differentiation, and (c) inhibition of L929 myoblast differentiation by a type I collagen layer occurs without a block in myogenin expression. Thus, the data suggest that inhibition of L929 myoblast differentiation by TGF-β1 may be accomplished by at least two mechanisms acting in concert. One mechanism leads to a block in the expression of myogenin whereas the other mechanism may involve TGF-β1-induced changes in cell adhesion that either block the action of myogenic differentiation gene products or prevent the function of other as yet unknown components of the myogenic differentiation pathway.

Transforming growth factors type β (TGF-β)1 are a group of homologous secretory polypeptides whose most notable biological actions are as paracrine inhibitors of cell proliferation, regulators of cell differentiation, and regulators of cell adhesion and extracellular matrix deposition (for reviews see Refs. 1 and 2). Expression of the three known mammalian TGF-β isoforms, TGF-β1, -2, and -3, is active throughout embryonic development and into adulthood. Expression of TGF-β1, -2, and -3 during embryogenesis occurs in discrete regions of many tissues with characteristic temporal patterns. Physiologically, TGFs-β are thought to participate as critical determinants of morphogenetic events during embryogenesis and tissue repair.

The differentiation of many cell types is markedly affected, induced, or repressed in response to TGFs-β (1, 2). Myoblast differentiation, in particular, is reversibly inhibited by the action of TGF-β1 and -2 on primary cultures and established lines of chick, rat, and mouse myoblasts (3–7). TGFs-β can suppress L929 myoblast differentiation if they are added before cells have reached the point of commitment to terminal differentiation (3). The mechanism by which TGFs-β inhibit myoblast differentiation is unknown, but it has been noted that an early and persistent response of myoblasts and many other cell types to TGF-β is a marked elevation in the expression of extracellular matrix components including fibronectin, collagen, proteoglycans, protease inhibitors, and cell adhesion receptors (8–14). The nature of the extracellular matrix and the ability of myoblasts to adhere to it can strongly influence myogenic differentiation (15–19). Thus, addition of fibronectin to myoblast cultures inhibits or alters the rate of differentiation (17, 18). Myogenic differentiation can also be disrupted by antibodies against extracellular matrix receptors of the integrin class (19).

An important family of myogenic differentiation genes includes MyoD1, myogenin, Myf-5, myd, and Myf-6/herculin (20–25). Their products, expressed during the differentiation process, induce cell commitment to a muscle phenotype. The MyoD1, myogenin, and Myf-5 products have a domain with predicted homology to the helix-loop-helix motif of c-myc. This structure is implicated in protein dimerization (20–22) involved in binding to certain muscle gene enhancers (26) and transcriptional activation of muscle-specific genes (27). Expression of myogenic differentiation genes might be the subject of regulation by factors that control myogenic differentiation such as TGF-β1 or cell adhesion components. Indeed, TGF-β1 can repress expression of MyoD1 in mouse myoblasts (7). However, the effects of an altered extracellular matrix on myogenic gene expression and their possible participation in the anti-myogenic action of TGF-β are unknown. Here, we provide evidence that TGF-β action on L929 rat skeletal muscle myoblasts might be accomplished by two parallel pathways that involve, respectively, decreased expression of the myogenic differentiation gene, myogenin, and changes in extracellular matrix that inhibits myoblast differentiation without blocking myogenin mRNA expression.

**EXPERIMENTAL PROCEDURES**

Cell Culture—L929 rat myoblasts (28) were grown in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum. To induce cells to differentiate subconfluent cultures were shifted to Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated horse serum (differentiation medium). In some experiments cell culture plastic was precoated 3–4 h with laminin or fibronectin (both 2 μg/cm², Collaborative Research) or with a 2-mm layer of fibrillar collagen (Vitrogen, Collagen Corp.). TGF-β1 was purified from bovine bone as described (29). It was used in all experiments in the concen-
washed twice with phosphate-buffered saline (pH 7.4) and then lysed in the presence of 50 mM glycylglycine buffer (pH 5.5) by freezing and thawing three times. Creatine kinase activity was measured from soluble cell extracts using a commercial kit (Sigma), and total protein was measured from the same samples using a Bio-Rad protein assay.

Northern Blot Hybridization—Total cellular RNA was prepared from L6E9 cells using the guanidine isothiocyanate/cesium chloride method as described (30), separated in formaldehyde/agarose gels, and transferred to nitrocellulose membranes. Cellular mRNA levels were measured using 32P-labeled cDNA probes specific to c-jun (EHJ-2, Ref. 31), junB (p655.20, Ref. 32), myogenin (pUC65-2, Ref. 21), and pro-α(1) collagen (pCG 116-a(I), Ref. 33). The mRNA level of a "housekeeping" enzyme glyceraldehyde-3-phosphate dehydrogenase was measured with a specific cDNA probe (pRGAPDH-13, Ref. 34) and used as the control.

Transfections—For these experiments, we used L6E9 cells that had been subcultured continuously for several months (>50 passages) and had lost the ability to spontaneously differentiate when shifted to differentiation medium ("late passage" cells). Sense (+) and antisense (−) myogenin-containing derivatives pEMSV.MGN+/- (21) of the expression vector pEMSVscribe (20) were obtained from Dr. Woodring E. Wright (University of Texas Southwest Medical Center, Dallas, TX). L6E9 cells were transfected using a modification of the calcium phosphate method (35). 96 h later cell cultures were fixed with 3.5% paraformaldehyde, permeabilized with 0.5% Triton X-100, and immunostained with myosin heavy chain-specific monoclonal antibody MF-20 (36) using rhodamine-conjugated anti-mouse IgG (Boehringer Mannheim) as secondary antibody. The number of myosin-expressing cells among 3–5 × 10⁵ cells was counted.

RESULTS AND DISCUSSION

The properties of the differentiating rat skeletal muscle myoblast cell line, L6E9, its requirements for differentiation, and its response to TGF-β have been reported (3, 28, 37, 38). Briefly, this cell line undergoes commitment, fusion, and biochemical differentiation when subconfluent monolayer cultures are shifted to mitogen-poor medium such as medium containing horse serum (28). The differentiation process of L6E9 cells can be prevented at the morphological and biochemical levels by the addition of picomolar concentrations of TGF-β1 or -β2 (3, 39). For inhibition of differentiation to occur, TGF-β must be added before L6E9 cells become committed to differentiate. Inhibition of differentiation lasts for as long as TGF-β is present in the medium and is reversible upon removal of the factor (3, 10). To investigate the mechanism of inhibition of L6E9 differentiation by TGF-β and the potential involvement of an altered extracellular matrix in this response, we examined the effect of TGF-β1 on the expression of genes whose products affect this differentiation process. TGF-β1 has already been shown to repress transcription of MyoD1 in mouse myoblasts (7). We could not detect expression of MyoD1 in L6E9 cells under any of the experimental conditions tested (not shown). However, differentiating L6E9 cells expressed myogenin mRNA during differentiation (Fig. 1A). A low level of myogenin mRNA was present in the cells before shifting them to mitogen-poor differentiation medium. Myogenin mRNA was elevated 10 h after the shift, reached approximately 15-fold over the basal level at 24 h (as determined by densitometry of Northern blots), and remained elevated for at least another 24 h (Fig. 1, A and B). TGF-β1 prevented this increase and maintained myogenin mRNA at the basal level (Fig. 1A).

Another set of genes relevant in myogenesis is the members of the jun family. Expression of v-jun in chick myoblasts prevents differentiation. The ability of Jun proteins to interfere with myogenesis may relate to their ability to associate with Fos proteins (40–42) that inhibit MyoD1 expression (43).

3 P. Vogt and H. Su, personal communication.
Certain cell lines respond to TGF-β1 with elevated junB and/or c-jun expression (44). Among the parameters studied here, the earliest changes observed after shifting LsE9 myoblasts to differentiation medium, LsE9 cells expressed junB and c-jun (Fig. 1C). In the absence of TGF-β1, the junB mRNA level increased transiently reaching 3-fold over basal 1 h after the shift, declined to an almost undetectable level by 5 h, and remained at this level for at least 20 h (Fig. 1, C and D). The addition of TGF-β1 at the time of shifting cells to differentiation medium rapidly potentiated the transient up-regulation of junB. Moreover, following the transient burst of junB expression, TGF-β1 action maintained junB mRNA at a relatively high level similar to that seen in cells before the shift to differentiation medium (Fig. 1, C and D). This effect was selective for junB since c-jun mRNA levels, which also decreased in differentiating cells, were unaffected by the addition of TGF-β1 (Fig. 1, C and D). The rapid response of junB preceded the 4-5-fold up-regulation of a1(I) collagen expression (Fig. 1, E and F) that is characteristic of LsE9 myoblasts and is similar in kinetics to the elevation of expression of other extracellular matrix components in response to TGF-β1 (3).

Given the prominent role that myogenin is believed to play in myoblast differentiation (21), the ability of TGF-β1 to prevent up-regulation of myogenin mRNA is likely to participate in the inhibition of LsE9 myoblast differentiation by this factor. However, additional mechanisms might be involved in the antimyogenic action of TGF-β1. This possibility was suggested by the observation that mouse fibroblasts rendered competent to differentiate by forced expression of MyoD1 mRNA failed to differentiate in the presence of TGF-β1 (7).

To duplicate this type of experiment in LsE9 cells, late passage cells that had a very diminished ability to spontaneously differentiate were transfected with the expression vector, pEMSV-MGN, that contains a rat myogenin gene under the transcriptional control of a constitutive SV40 viral promoter (21). The number of myosin heavy chain immunofluorescent cell clusters in cultures transfected with this plasmid was 5-7-fold higher than in cultures transfected with a control plasmid (Table 1). TGF-β1 inhibited the appearance of differentiated cells in pEMSV-MGN-transfected cultures (Table 1). These results suggested that TGF-β1 is able to block LsE9 myoblast differentiation by interfering with other events in addition to myogenin expression.

Since changes in the nature or composition of the extracellular matrix can inhibit myogenic differentiation and TGF-β1 elevates the expression of extracellular matrix components (Ref. 3, 8-14 and Fig. 1, E and F), we determined whether any of these extracellular matrix components could inhibit LsE9 myoblast differentiation. For this, LsE9 cells were plated in culture dishes that had been coated with fibronectin, laminin, or Vitrogen, a fibrillar collagen preparation containing predominantly type I collagen and a small amount of type III collagen. Under the conditions of our assay, fibronectin and laminin did not have any major effect on the ability of LsE9 cells to differentiate as determined by creatinine kinase activity assay, but cell plating on a layer of fibrillar collagen completely blocked biochemical differentiation of these cells (Fig. 2). The inhibitory effect of a collagen layer on LsE9 myoblast differentiation was also evident at the morphological level with no cell fusion being observed under these conditions (Fig. 3). Cells plated on collagen tended to aggregate, a morphological response that was much stronger in collagen-plated cells treated with TGF-β1 (Fig. 3). The marked morphological response of collagen-plated cells to TGF-β1 suggested that, in other cell types, other components of the cell adhesion apparatus in addition to collagen were altered in response to this factor. The preexisting collagen matrix might serve as a scaffolding for the retention and assembly of other LsE9 extracellular matrix components synthesized at high levels in

![Fig. 2. Effect of extracellular matrix proteins on LsE9 cell differentiation.](http://www.jbc.org/)

![Fig. 3. Effect of type I/III collagen on LsE9 myoblast cell fusion.](http://www.jbc.org/)
response to TGF-β1 (3, 8, 10, 11). In addition, TGF-β1 might increase the level or activity of as yet unidentified cell-cell adhesion receptors that contributed to the formation of cell clumps.

Interestingly, the level of myogenin mRNA in L6E9 cells plated on a collagen-rich matrix markedly increased after exposure to TGF-β for 24 h (Fig. 2) without a block in myogenic differentiation events other than myogenin expression. MyoD1 amplifies expression of its own gene (45). Whether the induction of a lower level of myogenin mRNA in collagen-plated myoblasts might reflect a block in the ability of myogenin to stimulate expression of its own gene is an interesting possibility.

These results indicate that myogenin expression alone may not be sufficient to induce L6E9 cell differentiation and that a collagen-rich matrix can inhibit differentiation by interfering with myogenic differentiation events other than myogenin gene expression. The data suggest that inhibition of myoblast differentiation by TGF-β1, and possibly other factors, may be accomplished by two mechanisms acting in concert. One of these mechanisms leads to a block in the expression of a myogenic differentiation gene, such as myogenin in L6E9 cells. The other mechanism is likely to involve effects mediated by changes in cell adhesion that either block the action of myogenin or prevent the function of other as yet unknown components of the myogenic differentiation pathway.

Effects on gene expression mediated via extracellular matrix adhesion receptors have been previously noted (19, 46). The mechanism by which cell adhesion to a collagen-rich extracellular matrix or the marked changes in cell adhesion induced by TGF-β1 transduce a stimulus that inhibits myoblast differentiation remains to be determined.

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