Functional Receptors for Transforming Growth Factor-β Are Retained by Biochemically Differentiated C2 Myocytes in Growth Factor-deficient Medium Containing EGTA but Down-regulated during Terminal Differentiation*

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Transforming growth factor-β (TGF-β) has been shown to block the morphological and molecular events associated with myoblast differentiation. During fusion of C2 myoblasts, TGF-β receptors are down-regulated, and muscle-specific genes become refractory to the inhibitory effects of TGF-β. To define further the mechanisms that modulate TGF-β receptor expression during myogenesis, we have developed culture conditions that support the differentiation of C2 cells in the absence of fusion and have examined the expression of functional TGF-β receptors in biochemically differentiated mononucleated myocytes. Exposure of C2 myoblasts to growth factor-deficient medium containing 1.4 mM (ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) leads to withdrawal from the cell cycle and high level expression of muscle-specific mRNAs and proteins. Under these conditions, TGF-β receptors fail to be down-regulated, and the differentiation program remains sensitive to repression by TGF-β. These studies demonstrate that EGTA uncouples muscle-specific gene expression from fusion in C2 cells and that in the absence of fusion, C2 myocytes retain a functional TGF-β signaling system.

Skeletal myoblasts provide an amenable system for analyzing the mechanisms whereby growth factors regulate cell proliferation and differentiation. In high concentrations (10–20%) of fetal bovine serum (FBS), myoblasts proliferate rapidly and do not express the muscle phenotype. Conversely, exposure of myoblasts to growth factor-deficient medium leads to cessation of cell division, fusion to form multinucleated myotubes, and expression of multiple muscle-specific genes (1–4). Previous studies have shown that FGF and TGF-β will substitute for serum and block activation of the muscle differentiation program (5–13). However, unlike the case with serum, which is highly mitogenic, suppression of differentiation by these purified growth factors does not necessarily involve stimulation of cell proliferation. Following fusion, myotube nuclei become refractory to the mitogenic effects of serum, and muscle-specific genes no longer respond to the negative effects of growth factors (4, 14). Little is known of the intracellular signals generated by growth factors to block myogenesis or of the mechanisms responsible for the loss of growth factor responsiveness in terminally differentiated muscle cells. Cell surface receptors for FGF, epidermal growth factor, and TGF-β have been reported to be down-regulated during differentiation of several rodent muscle cell lines, suggesting that modulation of growth factor receptors might contribute to the loss of growth factor responsiveness associated with myogenesis (15–18).

To explore further the relationship between myoblast fusion and TGF-β receptor expression, we have developed culture conditions that permit C2 myoblasts to differentiate in the absence of fusion. We report here that exposure of C2 myoblasts to growth factor-deficient medium containing 1.4 mM EGTA leads to withdrawal from the cell cycle and expression of muscle-specific mRNAs and proteins. Under these conditions, TGF-β receptors continue to be expressed at high levels, and these receptors remain functionally coupled to the intracellular signaling pathways that culminate in suppression of the myogenic phenotype. These observations are consistent with the conclusion that the decline in TGF-β receptors normally associated with terminal differentiation of C2 myoblasts is linked to fusion rather than to the induction of muscle-specific genes and may contribute partially to the lack of responsiveness of myotubes to the inhibitory effects of this growth factor.

MATERIALS AND METHODS

Cell Culture—The mouse muscle cell line C2 (19) was grown in DMEM containing 20% FBS (Hazelton Research Products, Denver, PA) as described previously (9). To initiate differentiation, media containing 20% FBS were replaced with DMEM containing 0.5% FBS or 10% dialyzed HS (GIBCO). To inhibit fusion, EGTA was included in the differentiation media at a final concentration of 1.4 mM. Calcium-free DMEM with 0.5% FBS or 10% dialyzed HS was also tested for the ability to stimulate differentiation of myocytes but was found to cause cell detachment within 2 or 3 days. The apparent toxicity of the latter media may reflect a requirement for low concentrations of calcium to remain viable. To obtain cultures enriched for myotubes, we transferred confluent cultures that had been exposed to DMEM with 10% HS for at least 3 days to DMEM with 0% HS plus 0.1 mM cytosine arabinoside for 2 days. TGF-β was obtained from R & D Products (Minneapolis, MN).
Creatine Kinase and Acetylcholine Receptor Assays—Creatine kinase was assayed as described previously (20). AchR was assayed by specific binding of [125I]-α-bungarotoxin to cell monolayers as described (21).

RNA Isolation and Northern Blot Hybridization—RNA was prepared from cells according to a modification of the 8 M guanidine HCl procedure (3). The relative abundance of individual mRNAs was determined by Northern blot hybridization, as described previously (12). Equivalent quantities of RNA from cells under each condition were applied to the lanes. The following probes were used: muscle creatine kinase, an Smal-EcoRI restriction fragment from plasmid R21 containing an insert homologous to canine muscle creatine kinase mRNA (22); glyceraldehyde-3-phosphate dehydrogenase, a 1640-base pair HhaI restriction fragment isolated from a chicken glyceraldehyde-3-phosphate dehydrogenase cDNA clone, pGAD-28 (23); troponin-T, an 800-base pair PstI fragment from a rat troponin-T clone (24); myogenin, an 1100-base pair EcoRI fragment from a mouse myogenin cDNA (25); MyoD1, a full-length mouse MyoD cDNA (26). DNA probes were labeled with [32P] by the method of Feinberg and Vogelstein (27).

Binding Assay for TGF-β—Cell surface TGF-β receptors were assessed on cell monolayer cultures in 24-well dishes. Cells were rinsed twice with binding buffer (DMEM containing 25 mM Hepes, pH 7.4, 0.1% bovine serum albumin) and then incubated in the same buffer at 37 °C for 2 h to remove possible cell-associated TGF-β. [125I]-TGF-β (2.5 Ci/mmol) was then added to a concentration of 100 pM with or without 2 nM unlabelled TGF-β. Two hours later, cells were washed four times with cold washing buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.1% bovine serum albumin) and twice with phosphate-buffered saline at 4 °C. [125I]-TGF-β was cross-linked to its receptor(s) with disuccinimidyl suberate (DSS) or bis(sulfosucinimidyl) suberate (BSS) (Sigma) at the indicated concentrations for 15 min. Cross-linking reactions were terminated by addition of 3 volumes of cold 10 mM Tris-Cl, pH 8, 1 mM EDTA for 5 min. The cells were then harvested, cell protein was determined, and equivalent amounts of protein from each condition were separated on denaturing SDS gels. Following electrophoresis, gels were stained with Coomassie Blue, destained, washed, dried, and exposed to x-ray film at -70 °C with intensifying screens. To quantitate relative levels of individual TGF-β receptor species, films were exposed for periods during which band intensity was linear with respect to time. Films were scanned with a densitometer, and the area under the peak corresponding to each receptor species was determined.

RESULTS

Differenitation of C2 Cells in Growth Factor-deficient Medium Containing EGTA—C2 myoblasts proliferate with a doubling time of about 12 h and do not express muscle-specific gene products when maintained in growth factor-deficient media containing EGTA. Although EGTA completely blocked myotube formation, high levels of muscle creatine kinase, troponin-T, and myogenin mRNAs were induced in mononucleated myocytes (Fig. 2). mRNAs for MyoD1, as well as glyceraldehyde-3-phosphate dehydrogenase, were expressed at equivalent levels in myoblasts, myocytes, and myotubes.

Previous studies with the rat L6E9 muscle cell line show that contractile protein mRNAs accumulated but were not translated in myoblasts maintained in differentiation media containing EGTA (37). Because of this disparity between muscle-specific mRNA and protein expression, we examined expression of muscle creatine kinase activity and cell surface Ach receptors in C2 myotubes and myocytes. As shown in Fig. 3, both muscle gene products were induced greater than 50-fold following transfer of undifferentiated myoblasts to growth factor-deficient media with EGTA. As observed for muscle-specific mRNAs, these muscle proteins were expressed in myocytes at lower levels than in myotubes. Together, these results show that C2 cells are able to express muscle-specific mRNAs and proteins in the absence of fusion when maintained in growth-factor-deficient media containing EGTA.

Measurement of TGF-β Receptors in Myoblasts, Myocytes, and Myotubes—Fusion of C2 cells is accompanied by a decline in cell surface receptors for TGF-β (18) and a concomitant loss of responsiveness to TGF-β (9). Direct binding assays have shown that the decrease in TGF-β binding in C2 myotubes, as opposed to myoblasts, is attributable primarily to a reduction in the number of cell surface receptors rather than to a decrease in receptor affinity for TGF-β (18). Having established conditions that supported the differentiation of C2 cells in the absence of fusion, we considered whether TGF-β receptors were down-regulated normally in differentiated mononucleated myocytes or whether the loss of these receptors was coupled to fusion. To compare the relative binding capacities of myoblasts, myocytes, and myotubes for TGF-β, we incubated monolayer cultures containing approximately equivalent numbers of cells from each condition with 200 pM [125I]-TGF-β, which has been shown to be sufficient to saturate all surface receptors (18). Cell-associated TGF-β was then cross-linked to its receptor(s) in the presence of the homobifunctional cross-linking reagent DSS, after which cross-linked species were resolved on denaturing SDS gels. Specificity of affinity labeling was assessed using a 20-fold excess of unlabeled TGF-β as a competitor.

C2 myoblasts possessed four major TGF-β-binding species with molecular masses of 250–300, 115, 85, and 65 kDa (Fig. 4). Following fusion, the four receptor species declined in abundance to varying extents. The 250–300-kDa species was down-regulated less than 2-fold following fusion and showed only a minimal decrease in expression in fusion-blocked myocytes. The 115-, 85-, and 65-kDa species declined dramatically following fusion (Fig. 4, A and B). A measurable level of these receptor species did persist, however, following fusion. The persistence of a low level of TGF-β receptors in myocyte cultures may be due, at least in part, to residual cells that were not incorporated into myotubes, since about 10% of cells failed to fuse. In contrast to their decline in myotubes, the 115- and 85-kDa species were down-regulated less than 2-fold in biochemically differentiated myocytes in differentiation medium containing EGTA (Fig. 4). The 85-kDa receptor reproducibly showed an altered migration in myocytes compared with myoblasts. The basis for this alteration is currently unknown. The 65-kDa species was down-regulated to a greater extent under these conditions but was expressed at a significantly higher level in fusion-blocked myocytes than in myo-
tube, where it was undetectable.

To confirm the specificity of DSS as a cross-linking reagent, the concentration dependence for cross-linking was determined and compared with that of a second water-soluble cross-linking reagent, BS3. As shown in Fig. 5, the four major TGF-β binding species were detected at all concentrations of DSS, and maximum amounts of these species were observed at 0.2 mM DSS. The same set of affinity-labeled complexes was generated by BS3. The affinity-labeled TGF-β-binding species are thus highly specific. A 25-kDa complex that was independent of cross-linker and was not competed by unla- beled TGF-β was often observed. We believe that this may represent unreduced homodimeric TGF-β.

Expression of Muscle-specific mRNAs and Proteins Remains Reversible in Fusion-blocked C2 Myocytes—We (9) and others (11, 13) have shown previously that TGF-β does not affect the expression of muscle-specific genes in terminally differ- entiated myotubes. Muscle genes also do not respond to serum stimulation following fusion2 (14). Having established that TGF-β receptors continued to be expressed in mononucleated myocytes in the presence of EGTA, we examined whether these receptors remained functionally coupled to the intracel- lular signaling pathways that lead to suppression of muscle- specific genes. As shown in Fig. 6, exposure of differentiated myocytes to TGF-β or to 20% fetal calf serum led to down-regulation of muscle creatine kinase mRNA and ACh receptors within 24 h. The more rapid decline of ACh receptor than muscle creatine kinase mRNA reflects the relatively short half-life of the receptor (21). Serum was more effective than TGF-β in down-regulating muscle-specific gene expression. This may have been due to multiple factors in serum that suppress myogenesis. By 3–4 days following growth factor stimulation, muscle creatine kinase mRNA declined nearly to basal levels in serum-stimulated cultures (data not shown).

For muscle-specific genes to be down-regulated in response to growth factor stimulation, it was important for cells to be at a low density (less than 20% confluency). At higher densities, the inhibitory effects of serum and TGF-β were diminished, particularly in the case of serum, which was highly mitogenic and led to increased cell density within 48 h. The apparent requirement for low density to observe maximum suppression of the myogenic phenotype is consistent with observations that C2 cells will begin to differentiate at high density, even when maintained in the presence of serum or TGF-β. This may be due to rapid depletion of growth factors from the media. Together, these results demonstrate that differentiated mononucleated myocytes retain cell surface receptors for TGF-β and other serum factors and that these receptors remain functionally coupled to the signaling cascade(s) that culminates in suppression of the myogenic phenotype.

DISCUSSION

EGTA Prevents the Down-regulation of TGF-β Receptors Normally Associated with Differentiation of C2 Myoblasts—It is well established that differentiation of skeletal myoblasts is triggered by the removal of exogenous growth factors and involves irreversible conversion to a postmitotic state, fusion, and permanent activation of muscle-specific genes (for review, see Ref. 4). Here we describe culture conditions that allow C2 myoblasts to differentiate without fusion. Using these culture conditions, we reached three principal conclusions regarding their differentiation. (a) Induction of muscle-specific mRNAs and proteins can occur in the absence of fusion. (b) TGF-β receptors are down-regulated in terminally differentiated

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2 J.-S. Hu and E. N. Olson, unpublished data.
FIG. 4. Affinity labeling of TGF-β receptors in C2 myoblasts (MB), myocytes (MC), and myotubes (MT). C2 myoblasts in growth medium were transferred to differentiation media for 5 days. Myocytes were maintained in differentiation medium containing EGTA. To obtain cultures enriched for myotubes, myoblasts were allowed to differentiate for 3 days and were then exposed to fresh medium containing cytosine arabinoside. Cell surface TGF-β receptors were measured by incubation of cell monolayers with 125I-TGF-β with or without unlabeled TGF-β, as indicated. After incubation, TGF-β was cross-linked to its receptors by incubation with 0.2 mM DSS (see "Materials and Methods"). Panel A, equivalent quantities of total cell protein from cultures under each condition were analyzed by electrophoresis on 10% SDS gels followed by autoradiography. The positions of the four major receptor species are indicated with arrows. Panel B, the autoradiogram in panel A was quantitated by densitometry (see "Materials and Methods"). Values for each receptor species are expressed relative to the maximal level of expression, which was assigned a value of 100%. The molecular mass of the 250-300-kDa species was determined from 5% polyacrylamide gels.

myotubes but remain at high levels in fusion-blocked C2 myocytes in growth factor-deficient media containing EGTA. (c) Muscle-specific gene expression remains susceptible to repression by serum factors and TGF-β in biochemically differentiated myocytes.

Direct binding assays and covalent cross-linking studies have shown that L6 and L6E9 myoblasts possess 65- and 85-kDa TGF-β receptor species (13, 18). In the current study, C2 myoblasts were shown to express four TGF-β receptor species with molecular masses of 250–300, 115, 85, and 65 kDa. TGF-β receptor complexes corresponding to the 250–300-, 85-, and 65-kDa species have been identified previously in other cell types and have been designated TGF-β receptor types III, II, and I, respectively (39–41). The 115-kDa TGF-β-binding species has not been studied extensively in other cell types and appears to be less widespread in its cell-type distribution. Although the precise functions of these receptors remain unknown, it seems unlikely that the 250–300- and 115-kDa species in C2 cells mediate the actions of TGF-β on myogenesis, since these forms of the receptor are absent from L6E9 myoblasts, which are also prevented from differentiating by TGF-β (13).

Following fusion of C2 myoblasts, all four receptor species were down-regulated to varying degrees. TGF-β receptors have also been reported to be down-regulated during differentiation of L6 myoblasts (18). It remains to be established whether a common mechanism mediates the down-regulation of TGF-β, epidermal growth factor, and FGF receptors during differentiation of these and other muscle cell lines (15–18). In this regard, we have noted that during fusion of C2 cells, FGF receptors are down-regulated to a greater extent than the four TGF-β receptor species.1 In contrast to the decline in TGF-β receptors observed during terminal differentiation of C2 and L6 myoblasts, the L6E9 cell line that was derived from L6 retains TGF-β receptors following fusion (13). That muscle-specific genes in L6E9 myotubes do not respond to TGF-β (13) suggests that certain aspects of the TGF-β signaling pathway may become nonfunctional following fusion. This also implies that the loss of TGF-β receptors which

1 G. Spizz, J.-S. Hu, and E. N. Olson, unpublished data.
Monolayers of undifferentiated myoblasts were incubated with 100 pM TGF-β. After washing to remove unbound TGF-β, monolayers were exposed to various concentrations of DSS or BS3, and labeled species were resolved on 10% SDS gels followed by autoradiography. Protein determinations were performed on each sample, and equivalent quantities of protein from each sample were applied to the gel.

accompanies fusion of L6 and C2 myoblasts may not solely account for the inability of these cells to respond to the inhibitory effects of TGF-β.

Differentiation of C2 Cells Is Reversible in the Presence of EGTA—Previous studies of the relationship between fusion and the expression of muscle-specific gene products have yielded contrasting results. Avian and fetal calf myoblasts, for example, express muscle-specific mRNAs and proteins as mononucleated cells when fusion is inhibited by EGTA (30-36, 38). Mouse MM14 and C2C12 myoblasts also can express muscle-specific gene products in the absence of fusion (15), although their behavior in medium containing EGTA has not been characterized previously. In contrast, rat skeletal muscle cells transcribe muscle-specific mRNAs but fail to express the corresponding proteins in the presence of EGTA (37). Because of the sensitivity of myoblasts to subtle differences in culture conditions and the apparent species-specific behavior of myoblasts in culture, the properties of an individual muscle cell type cannot be inferred from results obtained in a different species under different culture conditions. The unique properties described for different muscle cell types probably reflect different origins of muscle cells (42) as well as unique regulatory programs (see Refs. 25, 26, and 43) or may also reflect subtle variations in culture conditions.

Using mouse MM14 myoblasts, Hauschka and co-workers (2) have shown that irreversible commitment to the postmitotic state begins 2-3 h after removal of mitogens, with greater than 95% of the population becoming postmitotic by 12 h. Irreversible withdrawal from the cell cycle precedes the loss of cell surface FGF and epidermal growth factor receptors and fusion (15, 16). These results suggest that a postreceptor mechanism may be responsible for the initial loss of responsiveness of muscle cells to growth factors. C2C12 muscle cells have also been shown to exhibit a total loss of FGF receptors and to acquire a postmitotic phenotype in mitogen-poor medium, irrespective of whether they are mononucleated or multinucleated (15). That C2 cells retain the ability to respond to serum factors and TGF-β following differentiation in the presence of EGTA indicates that under these conditions, at least a subset of cell surface receptors remains functionally coupled to the intracellular pathways that suppress myogenesis. Although the mechanisms by which growth factors suppress myogenesis remain unknown, the observation that muscle gene products were able to be down-regulated in the presence of EGTA suggests that the signaling pathway through which growth factors suppress myogenesis may not require high concentrations of extracellular calcium.

The results of this study are consistent with studies by Konigsberg and co-workers (29, 38), who have shown that primary quail myoblasts express muscle-specific genes in mitogen-deficient media containing EGTA and that stimulation of fusion-blocked myocytes with serum results in rapid down-regulation of myosin heavy chain and α-actin mRNAs. Down-regulation of these mRNAs precedes reentry of myocytes into the cell cycle and appears to be due, at least in part, to accelerated decay of muscle-specific mRNAs. Similarly, using the rat muscle cell line ts3b-2, which is temperature-sensitive for commitment to terminal differentiation, Nguyen et al. (44) showed that differentiation was reversible following mitogen stimulation at a nonpermissive temperature. The behavior of C2 cells in growth factor-deficient medium with EGTA also resembles that of the B&H1 muscle cell line in several respects. B&H1 cells, for example, express muscle-specific genes following exposure to mitogen-deficient media but fail to fuse (3, 5, 8-10, 12, 21, 45, 46). TGF-β receptors also remain at high levels following differentiation of B&H1 cells, and muscle-specific gene expression is reversible (3, 5, 8, 9, 12, 18, 21, 45).

Modulation of growth factor receptors provides a mechanism whereby a target cell can regulate its growth factor
responsiveness. In certain muscle cell types such as C2, the decline in TGF-β receptors which accompanies fusion may contribute to their commitment to a differentiated state (see Ref. 15). It should be emphasized, however, that additional events must also contribute to irreversible commitment, since TGF-β receptors are not down-regulated during differentiation of all muscle cell types (13). Myoblasts represent the only system described thus far in which TGF-β receptors have been shown to be regulated during terminal differentiation. Since TGF-β regulates the differentiation of a wide variety of cell types, it will be interesting to determine whether TGF-β receptors are modulated during differentiation of other cell types and whether this type of regulation could be responsible for alterations in sensitivity to TGF-β under various cellular states.

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