Effects of Insulin-Like Growth Factors and Transforming Growth Factor-β on the Growth and Differentiation of Muscle Cells in Culture (43059)

DAINA Z. EWTON AND JAMES R. FLORINI
Biology Department, Syracuse University, Syracuse, New York 13244

Muscle cells growing in tissue culture provide a good model with which to study the mechanisms involved in the processes of skeletal muscle growth and differentiation. Mononucleated myoblasts first undergo cell division and then, under appropriate conditions, fuse into terminally differentiated myotubes. Upon fusion, DNA synthesis ceases and coordinate expression of muscle-specific proteins begins. We have attempted to identify the growth factors and hormones that regulate the processes of myoblast proliferation and terminal differentiation, and to further characterize the mechanisms involved in these processes.

Stimulatory Effects of Insulin-Like Growth Factors

Early in our studies we concluded that the somatomedins or insulin-like growth factors (IGF) were the most potent anabolic agents for skeletal muscle cell growth (1, 2). The IGF are polypeptide growth factors that are thought to mediate many if not all of the actions of growth hormone. There are two forms of IGF, IGF-I and IGF-II, and there are specific cell surface receptors for each. The Type I IGF receptor, a large protein (molecular mass > 300 kDa), consists of two α and two β subunits and has the highest affinity for IGF-I; however, it will also bind IGF-II and insulin, but with a much lower affinity (3, 4). The Type II receptor is a single chain large protein (molecular mass 220 kDa) which binds IGF-II with the highest affinity, may or may not bind IGF-I, and does not bind insulin. Recently, the Type II receptor has been shown to be identical to the cation independent mannose-6-phosphate receptor (5, 6). Our early studies with rat IGF-II (7) showed that the suppression of protein degradation and stimulation of amino acid uptake were much more sensitive to the hormone than the stimulation of proliferation and differentiation, suggesting that the Type II receptor mediated the former, and the Type I receptor the latter, effects of the IGF. In characterizing these actions of the IGF on skeletal muscle cells in culture, we also wanted to determine which of these receptors mediated each of the various actions.

We first compared the relative potency of the different forms of IGF and insulin on rat L6 skeletal muscle cells. As shown in Figure 1, human IGF-I was the most potent of the IGF in all four of the processes we studied: stimulation of amino acid uptake, decrease in protein degradation, increase in cell proliferation, and stimulation of differentiation as measured by the increase in creatine kinase activity (1). In each case it required approximately 3–10 times more IGF-II to get the same response, and insulin was the least potent of all. From these results we concluded that the Type I IGF receptor probably mediated all of these actions, since the relative potencies of the growth factors reflected the specificity of the Type I receptor.

That the responses to IGF and insulin were mediated by the Type I receptor was further confirmed when we studied the effects of a recombinant DNA analog of IGF-I, (Thr59-IGF-I). Thr59-IGF-I had equal biologic activity to the human IGF-I in all four of the processes we examined. However, when we compared the affinity cross-linking of human IGF-I and Thr59-IGF-I to L6 myoblast monolayers in culture, we found that human IGF-I apparently bound to both Type I and Type II receptors, but Thr59-IGF-I bound only to the Type I receptor (1). These results provided further evidence that the Type I receptor mediated the actions of IGF in muscle cells.

The stimulatory effects of IGF on skeletal muscle growth and differentiation are seen not only in rodent muscle cell lines, but also in primary avian skeletal muscle cultures. Schmid *et al.* (8) have shown stimu-

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lation of differentiation of chick primary myoblasts by IGF-I. Experiments in our laboratory designed to determine which IGF receptor mediated the actions of IGF in quail myoblasts yielded unexpected results. Using affinity cross-linking of IGF-I or IGF-II to monolayer cell cultures, we could not detect a cell surface Type II receptor on any avian cells (chick embryo fibroblasts, chick aorta smooth muscle cells, chick lung fibroblasts, quail skeletal muscle cells) (unpublished data). Figure 2 compares affinity cross-linking of rat IGF-II to rat L6 myoblasts and quail primary muscle culture monolayers. Whereas in L6 myoblasts binding of rat IGF-II, which is not displaced by insulin, is primarily to the Type II receptor; in the quail IGF-II binds only to the Type I receptor α subunit (135 kDa). Binding to the large >200-kDa band is completely displaced by insulin; therefore, this is not a Type II receptor but most likely represents the cross-linking of two α subunits of the Type I receptor. Thus, in quail muscle cultures (and presumably other avian muscle cultures (9)) the effects of IGF also appear to be mediated by the Type I receptor.

Inhibition of Differentiation by Transforming Growth Factor-β

Early in our studies with IGF we had to purify our own IGF-II from Buffalo rat liver cell-conditioned medium. We discovered that this medium also contained a substance that inhibited the differentiation of muscle cells in culture (10). We have subsequently shown that this differentiation inhibitor was identical to transforming growth factor-β (TGF-β) (11). TGF-β is a very potent inhibitor of myoblast differentiation and inhibits all aspects of differentiation: myoblast fusion into myotubes as well as all measured aspects of biochemical differentiation—increase in creatine kinase activity, acetylcholine receptors, myosin heavy chain, and α-actin (11–13).

Figure 3 shows that the addition of TGF-β (1 ng/ml) to L6 myoblasts completely inhibited the increase in creatine kinase activity, but that the inhibitory effect is reversible if the TGF-β is removed or if fresh TGF-β is not added to cells cultured for extended periods of time. TGF-β-treated myoblasts assume a characteristic morphology; the cells contain extensive stress fibers that are composed of β- and γ-actin filaments (14). Our earlier studies with the differentiation inhibitor from BRL cells showed that treatment of quail primary muscle cells with this crude form of TGF-β caused increased secretion of Type I collagen from the unfused myoblasts (7). This has been confirmed by Massague *et al.* (12) who showed that not only several types of collagen but also fibronectin were secreted from TGF-β-treated rat muscle cells.

In most of our experiments, we have stimulated differentiation of muscle cells by the addition of IGF-I (10–20 ng/ml) or high levels of insulin (0.3 μM) which acts through the Type I IGF receptor in serum-free medium. To assess the specificity of the effects of TGF-β on differentiation, we examined TGF-β actions on the stimulation of other processes by IGF-I. TGF-β had no effect on IGF-I stimulated L6 myoblast proliferation or suppression of proteolysis, but when added in combination with IGF-I, TGF-β stimulated amino acid uptake in an additive manner (14). When we looked at the binding of IGF-I to cell surface receptors, we found that the addition of TGF-β had no effect on binding of IGF-I to either the Type I or Type II IGF receptors (14). Thus, TGF-β appears to act at several steps removed from IGF receptor binding.

Binding studies using ^{125}I-labeled TGF-β confirmed that L6 myoblasts contain specific receptors for TGF-β which do not cross-react with IGF-I, IGF-II, insulin, or epidermal growth factor. Affinity cross-linking experiments revealed binding of TGF-β to two proteins, 65 kDa and 85 kDa (15, 16); we could not detect any binding to the large >280-kDa receptor present on many other cell types (17). Figure 4 shows binding of TGF-β to L6 myoblasts as they differentiate into myotubes. It can be seen that as myoblasts fuse into myotubes (accompanied by an increase in creatine kinase activity), TGF-β binding to the muscle cultures decreases. No TGF-β binding could be detected by Day 4 after insulin addition when more than 90% of the cells were in myotubes. This loss of TGF-β receptors was reflected in a loss of responsiveness of differentiated L6 myotubes to TGF-β; although TGF-β stimulated α-aminoisobutyric acid uptake in L6 myoblasts, it had no effect on α-aminoisobutyric acid uptake in myotubes (16). A similar phenomenon has been reported by Olwin and Hauschka (18) who saw a decrease in epidermal growth factor and fibroblast growth factor receptors on mouse MM14 muscle cells as they differentiated. The loss of TGF-β receptors with differentiation seems to be the *result* of terminal differentiation and *does not cause* differentiation. This view is supported by the fact that a nonfusing mouse myoblast cell line, BC3H1, undergoes reversible differentiation, and does not lose TGF-β receptors upon differentiation (16).

Our early experiments examining the time course of TGF-β action showed that TGF-β acted at an early point in the differentiation pathway of rat L6-A1 myoblasts to inhibit the process (10). Subsequently, Massague *et al.* (12), and Olson *et al.* (13), also demonstrated that TGF-β was ineffective if added to L6-E9 or C2 myoblasts after the cells became committed to differentiation. Thus, it appears as if TGF-β is an inhibitor of the process of *commitment* to terminal differentiation, but has no effect on the *expression* of differentiated functions. We are currently attempting

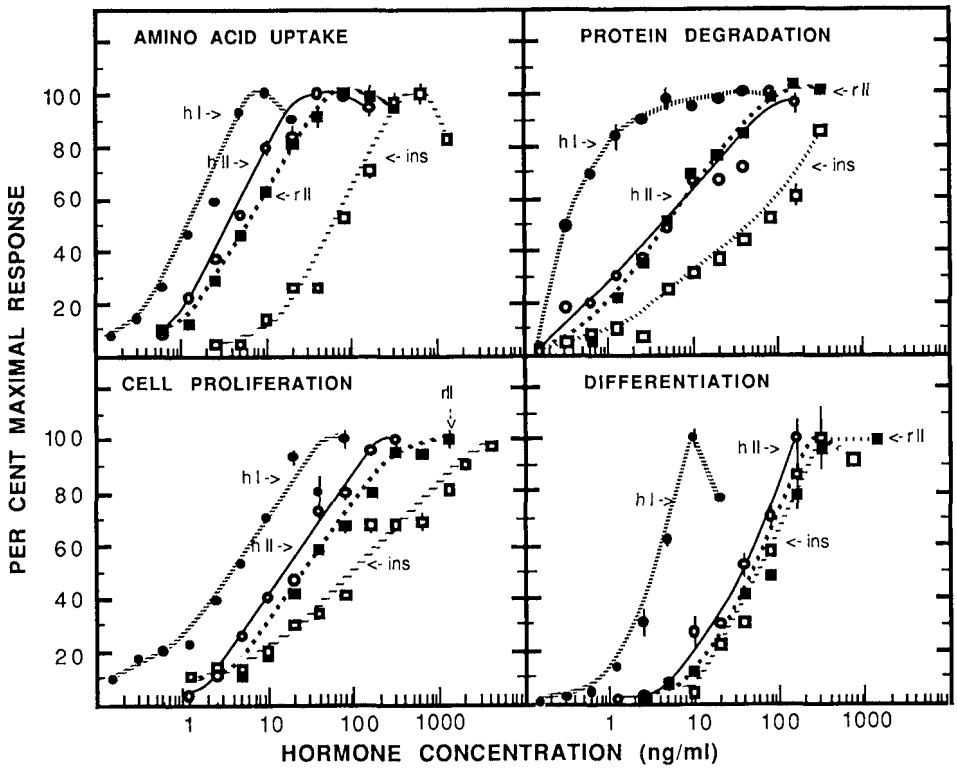

Figure 1. Actions of IGF and insulin on L6 myoblasts. Human IGF-I (hI), human IGF-II (hII), rat IGF-II (rII), and insulin (ins) were added at the indicated concentrations in serum-free Dulbecco's modified Eagle's medium to L6 myoblasts, and effects were measured at the following times after hormone additions: α-aminoisobutyric acid uptake, 4 hr; inhibition of protein degradation, 4 hr; cell proliferation, 24 hr; differentiation (creatine kinase activity/mg DNA), 72 hr. The results are the mean ± SE for triplicate determinations (reproduced with permission from Reference 1).

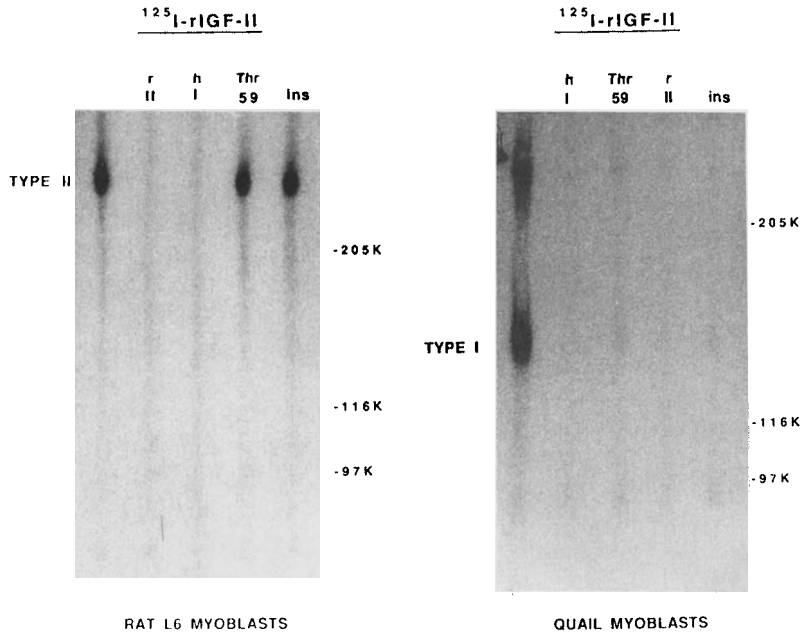

Figure 2. Affinity cross-linking of ^{125}I-labeled rat IGF-II to rat L6 myoblasts and primary quail myoblasts. ^{125}I-Labeled rIGF-II was cross-linked in the presence of disuccinimidyl suberate to L6 myoblasts (left) or quail myoblasts (right) in the absence (first lane) or presence of 300 ng/ml of unlabeled hIGF-I, Thr59-IGF-I, rIGF-II, or 10 μg/ml insulin. The cells were solubilized in sodium dodecyl sulfate under reducing conditions in the presence of dithiothreitol, and receptors were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

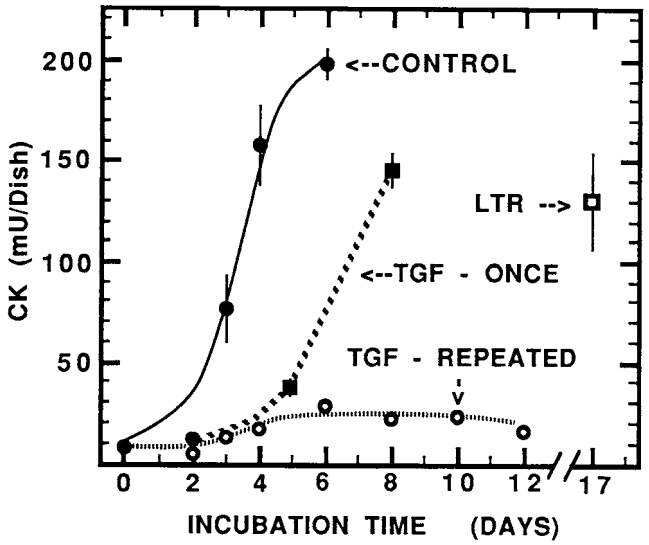

Figure 3. Persistence and reversibility of TGF-β inhibition of myoblast differentiation. L6 myoblast cultures were treated with insulin (0.3 μM) or insulin plus TGF-β (1 ng/ml) and creatine kinase activity was determined at the indicated times. Control cultures received only insulin at time zero. TGF-Once cultures received TGF-β plus insulin only at zero time. TGF-Repeated cultures received fresh TGF-β and insulin at 48-hr intervals. LTR indicates the long-term reversal of inhibition that was observed when TGF-β was removed after 8 days in culture, fresh insulin was added, and the cultures were allowed to differentiate until Day 17. Data are mean ± SE of triplicate determinations (reproduced with permission from Reference 11).

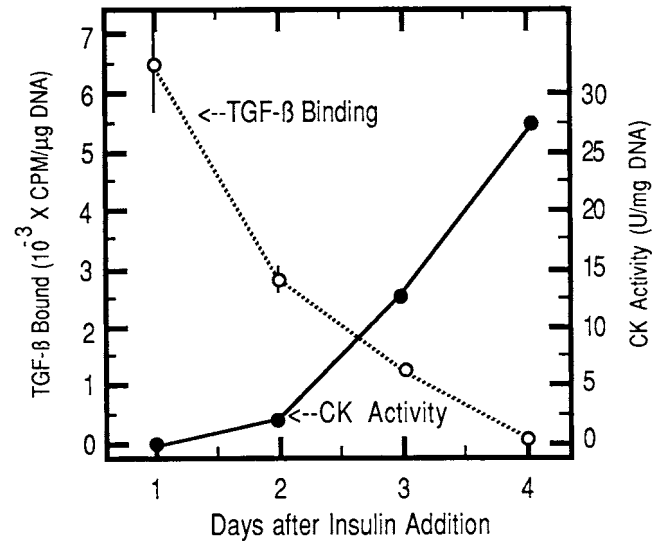

Figure 4. Time course of differentiation and loss of TGF-β receptors in L6 myoblasts. At zero time, L6 myoblast monolayers were washed and incubated with Dulbecco's modified Eagle's medium containing 1% horse serum and 0.3 μM insulin to stimulate differentiation. At the indicated times, parallel cultures were removed for creatine kinase (CK) analysis and for measurement of ^{125}I-labeled TGF-β binding. Results are mean ± SE of triplicate determinations (reproduced with permission from Reference 16).

to use this action of TGF-β, in tandem with previously described inhibitors of muscle differentiation, to dissect the processes involved in the differentiation pathway.

Conclusion

Thus, we have identified and begun to characterize the actions of two growth factors which appear to play major roles in controlling the growth and differentiation of muscle cells in culture, IGF-I and TGF-β. The IGF stimulate both proliferation and differentiation of muscle cells, and TGF-β is a very potent inhibitor of all aspects of myoblast differentiation. These two growth factors are active not only in mammalian systems, but also appear to modulate the development of avian muscle cells. Studies incorporating the use of IGF-I and TGF-β along with other purified growth factors in serum-free defined medium should yield promising information on the control mechanisms involved in skeletal muscle growth and differentiation.

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