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# Activin A Inhibits Differentiation of Chick Myogenic Cells *In Vitro*

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**ABSTRACT**—We investigated the effect of activin A on the *in vitro* differentiation of primary myogenic cells isolated from chick embryonic breast muscle. As judged by the morphology of the cells and accumulation of creatine kinase, activin A inhibited myogenesis in a dose-dependent manner. The inhibitory activity was reversibly suppressed by follistatin. Activin A also inhibited the expression of MyoD1. Our data also suggest that the inhibitory activity of activin A is exerted neither on proliferation of myoblasts or the growth of myotubes, but rather an early phase of differentiation following the withdrawal from cell cycle. A comparison of actions of activin A and TGF- $\beta$  suggests that points of action is similar.

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## INTRODUCTION

Developmental biologists are interested in the skeletal muscle formation because it involves many of the major themes of developmental biology; cell lineage determination, migration, commitment, cell-cell interaction, fusion, differentiation, cell growth, maturation, and morphogenesis. Many of the previous studies *in vitro* have revealed that humoral factors, especially growth factors such as fibroblast growth factor, play primary roles in regulating myogenic differentiation (for review see Florini and Magri, 1989).

Recently, transforming growth factor (TGF)- $\beta$  has also received much attention as one of the myogenesis-regulating factors. TGF- $\beta$  is a potent inhibitor of myogenic differentiation and has been suggested to act at the commitment stage of myogenic differentiation, but to have no effect on myoblast proliferation. According to Florini and Magri (1989), “commitment to myogenic differentiation” refers to the first part of myogenic differentiation, in which proliferating myoblasts are converted to postmitotic cells that are irreversibly committed to subsequent myogenic differentiation, but have not yet begun to fuse or to exhibit the other properties of terminally differentiated muscle cells.

Activins are members of TGF- $\beta$  superfamily. These molecules are multifunctional and have been known to regulate various cell functions. Activins are currently regarded as differentiation factors, since they have been shown to modulate the differentiation of many types of cells (for review see Massagué, 1990).

It is well known that activins induce the formation of

mesoderm during early embryonic development (for review see Asashima, 1994). *In vitro*, activins can induce the formation of mesodermal cells such as muscle cells in a concentration-dependent manner. An activin-binding protein, follistatin, has also been identified. Follistatin acts as an antagonist of activin *in vitro*, whereas the *in vivo* function of follistatin is currently unclear (Massagué, 1990).

TGF- $\beta$  has also been shown to be a mesoderm-inducing factor (Smith, 1993; Asashima, 1994). Thus, it is of considerable interest that TGF- $\beta$  has dual activities of inhibition and stimulation of the development of myogenic cells. Since the effect of activins on myogenic differentiation has not been investigated so far, we decided to examine the effect of activin A on *in vitro* myogenic differentiation and to compare it to that of TGF- $\beta$ .

In this paper, we investigated the effect of activin A on the *in vitro* differentiation of primary myogenic cells isolated from chick embryos. We demonstrate that activin A dose-dependently inhibits myogenic differentiation. The inhibitory activity is comparable to that of TGF- $\beta$ , suppressing the transition from the proliferation phase to the differentiation phase of myogenic cell development.

## MATERIALS AND METHODS

### Reagents

Human recombinant activin A and human recombinant follistatin were gifts from Dr. Eto (Ajinomoto Co. Inc.). Eagle's minimum essential medium (MEM) was from Nissui (Tokyo). Horse serum was from Kojinbio (Tokyo). Chick serum was from Gibco. Transferrin (Tf: ovotransferrin iron-complex type II from chicken egg white) was obtained from Sigma.

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### Cell culture

Preparation and culture of primary myogenic cells from breast muscle of 11-day chick embryos were carried out according to the procedure of Kimura *et al.* (1982). Cells were cultured at  $3 \times 10^5$ /dish in 1.5 ml of culture medium (85% MEM - 15% horse serum - 30  $\mu$ g/ml Tf) in gelatin-coated 35-mm dishes (Falcon) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The culture media were not changed during the incubation.

### Creatine kinase assay

Creatine kinase (CK) activity in cell lysates was analyzed according to Kimura *et al.* (1985), using a diagnostic kit (IATRON) and autoanalyzer (Simadzu CL-7100). The CK activity (mU/dish) was expressed as an average of three dishes.

### Northern blot analysis

The expression of MyoD1 was examined by Northern blot analysis according to Momoi *et al.* (1992). In this analysis, cells were cultured at a cell density of  $1 \times 10^7$ /dish in 10 ml of MEM containing 15% horse serum and 2% of chick serum in gelatinized 100-mm tissue culture dishes (Corning).

## RESULTS

### Inhibitory effect of activin A on myogenic differentiation

The effect of activin A on myogenic differentiation was examined morphologically and biochemically. Control cells show well-developed myotubes after 96 hr in culture (Fig. 1A). In contrast, cells treated with 100 ng/ml (approximately 4 nM) activin A from the beginning of cultivation formed only thin myotubes (Fig. 1B). For comparison, the effect of TGF- $\beta$ 1 was also examined at 100 ng/ml, and it was confirmed that TGF- $\beta$ 1 also suppressed myogenic differentiation (data not shown). The activin A and TGF- $\beta$  were comparable in their abilities to inhibit myogenic differentiation. Consistent with the morphological observations, activin A was also shown to inhibit biochemical differentiation. As shown in Fig. 2A, activin A dose-dependently inhibited CK accumulation, a biochemical marker of myogenic differentiation.

We examined how the inhibitory effect changed when the starting time of activin A-treatment after plating was changed.

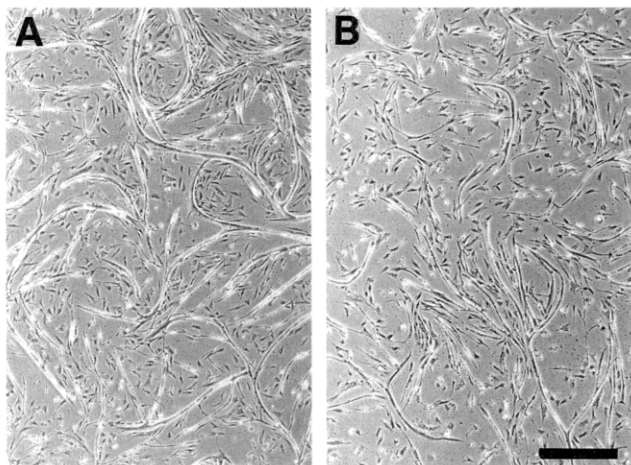


Fig. 1. Chick myogenic cells cultured for 96 hr in control medium (A) and 100 ng/ml activin A-containing medium (B). Bar: 500  $\mu$ m.

Activin A (100 ng/ml) was added to the cultures 0, 24, 48, 72, and 96 hr after plating and the cells were harvested after 120 hr (Fig. 2B). When activin A was provided from the beginning of cultivation, essentially no CK accumulation was observed. Similarly, if activin A-treatment was begun 24 hr after plating, CK accumulation was almost completely inhibited. In the case of treatment at 48 hr, CK accumulation was rather high although significantly less than the untreated control. When activin A was added at 72 or 96 hr, the reduction in CK activity was very low.

We further examined the effect of activin A in more detail by subdividing the starting time of activin-treatment around 48 hr after plating. As shown in Fig. 3B, the activin-susceptibility was fairly discrete between 40 and 48 hr.

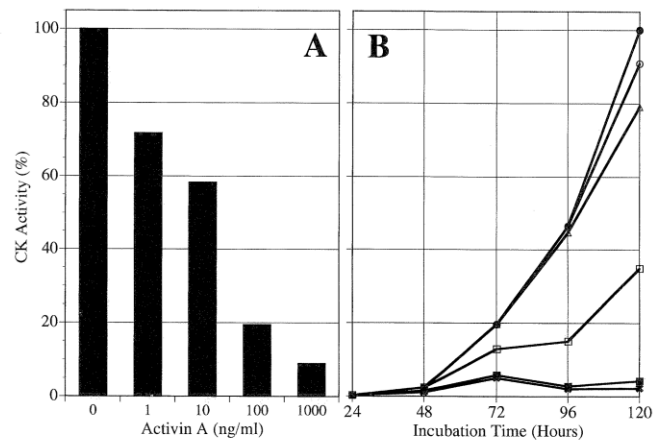


Fig. 2. Effect of activin A on myogenic differentiation. (A) Dose-response for CK accumulation. The cells were cultured for 120 hr. (B) Effect of activin A given at various times after the beginning of cultivation on CK accumulation. Activin A (100 ng/ml) was given at 0 (x), 24 (■), 48 (□), 72 (△), and 96 hr (○) after seeding. Control (●) was cultured in the absence of activin A. Each plot is the mean of three independent measurements.

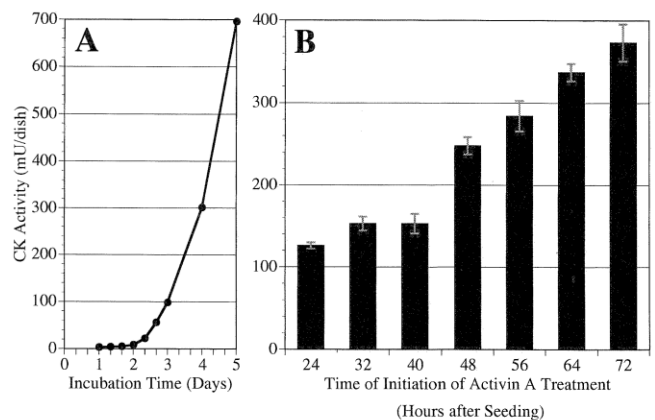


Fig. 3. Effect of activin A on myogenic differentiation. (A) Time course of CK accumulation of control culture. (B) CK activity of cultures treated with activin A (100 ng/ml) from various times after the beginning of cultivation.

### Effect of follistatin on inhibitory activity of activin A

The effect of follistatin on the myogenesis-inhibitory activity of activin A was examined. Follistatin (1 µg/ml) could cancel approximately 80% of the inhibition by activin A (100 ng/ml) when added to the cultures at a 7:1 molar ratio of follistatin:activin A (Fig. 4A). This effect of follistatin was also shown to depend on the time of the treatment during cultivation. The data in Fig. 4B show that the cells that had been treated with activin A from the beginning of the cultivation could differentiate if treated with follistatin at any time during the cultivation, i.e., the cells began to differentiate in a delayed manner.

### Effect of activin A on the expression of MyoD1 in myoblasts

We examined the effect of activin A on the MyoD1 expression in myoblasts. Figure 5 shows that activin A inhibits the expression of MyoD1.

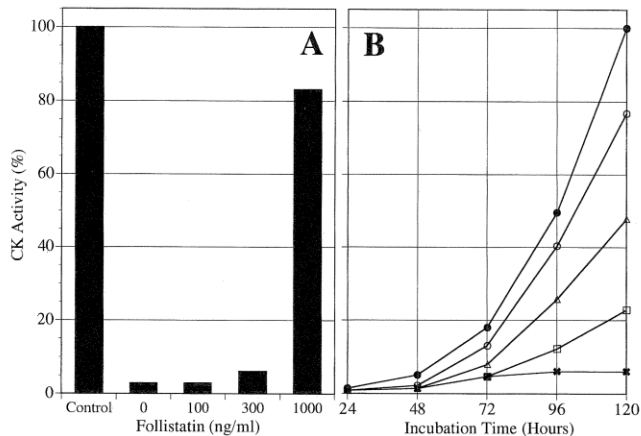


Fig. 4. Effect of follistatin on myogenesis-inhibitory activity of activin A. (A) Reversal by follistatin of activin A (100 ng/ml)-treated cells from inhibition of myogenic differentiation. Control means the culture not treated with activin A and follistatin. Others were cultured in the presence of 100 ng/ml activin A and added follistatin of various concentrations. (B) Effect of follistatin (1 µg/ml) added to activin A (100 ng/ml)-treated cells (●) at 24 (○), 48 (△), and 72 hr (□) after seeding. Control (●) was cultured in the absence of both activin A and follistatin. Each plot is the mean of three independent measurements.

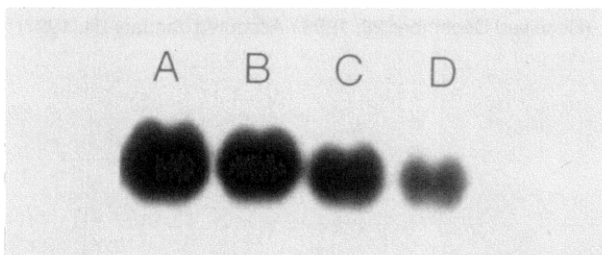


Fig. 5. Effect of activin A on the expression of MyoD1. Total RNA (20 µg) extracted from myogenic cells at 0 (A), 6 (B), 12 (C), and 24 hr (D) after plating and exposure to activin A (1 ng/ml) was hybridized to a chick MyoD1 probe.

## DISCUSSION

In this paper we have shown that activin A dose-dependently inhibits the *in vitro* differentiation of primary myogenic cells isolated from chick embryos (Figs. 1 and 2A). The dose-dependency of their inhibition is comparable to that observed for TGF-β-mediated inhibition of myogenesis *in vitro*.

The extent of myogenesis depends on several processes, including proliferation of myoblasts, withdrawal from cell cycle, cell fusion, and growth and maturation of myotubes. To determine the step(s) at which the inhibitory action of activin A was exerted, we did some experiments.

First, we examined how the myogenic differentiation is affected when the cells were treated with activin A (100 ng/ml) 0, 24, 48, 72, and 96 hr after plating (Fig. 2). When the cells were treated with activin A within 24 hr, CK accumulation was almost completely suppressed. However, when treated at 48 hr, CK accumulation occurred, although it was considerably lower compared to control cultures. If activin A-treatment was performed at 72 or 96 hr, only a little inhibition was observed. In our culture system, untreated control cultures do not produce significant amounts of CK at 24 hr, but a small amount of accumulation is observed at 48 hr (see Fig. 2B). Although the cellular synchronicity during the differentiation process is not high, these observations suggest that commitment from proliferation phase to differentiation phase occurs between 24 and 48 hr after plating and a small fraction of the cell population is beginning to fuse at 48 hr. The small amount of CK accumulation observed in the cultures treated with activin A at 0 or 24 hr is possibly due to a cell population that had already become committed to differentiation *in vivo* or during the early period of culture *in vitro*. At 48 hr, a considerable fraction of, and at 72 hr, most of the cells, have become committed and are able to differentiate in the presence of activin A. Thus, these results imply that the inhibition of myogenesis by activin A does not occur during the myotube growth phase, but rather during the proliferation or/and commitment phases of myogenic cells. Activin A had no effect on myoblast proliferation (data not shown).

These results strongly suggest that the inhibition of myogenic differentiation by activin A is possibly due to the inhibition of an early phase of differentiation, that is the step between cell cycle withdrawal and commitment to differentiation. This is also suggested by the sharp difference in activin-sensitivity before and after 48 hr (Fig. 3B). Since TGF-β has previously been suggested to inhibit the commitment of myoblasts, it is possible to speculate that activin A and TGF-β exhibit similar effects on myogenic differentiation. In preliminary experiments activin A and TGF-β cotreatment was shown to inhibit myogenic differentiation in an additive manner.

We examined how the inhibitory activity of activin was affected by follistatin, which is known to bind specifically to activin and inhibit its biological activities. As can be seen in Fig. 4A, the inhibitory activity of 100 ng/ml activin A was repressed only slightly by follistatin at concentrations of less

than 300 ng/ml, but 80% or more of activin's inhibitory activity was repressed at 1 µg/ml. The molar ratio of 100 ng/ml activin : 1 µg/ml follistatin is some 1:7. This result agrees with previous observation that the bioactivity of activin in erythroid differentiation system of Friend cells is suppressed by follistatin at molar ratios of 5 or more (Eto, personal communication).

When follistatin (1 µg/ml) was added at various times to cultures containing 100 ng/ml activin A, all the cultures were released from the activin-induced inhibition and delayed CK accumulations were observed, although the time courses were different depending on the time of the follistatin treatment (Fig. 4B). The fact that activin A inhibits the MyoD1 expression (Fig. 5) suggests the reduction of differentiative potential during the activin-induced arrest of commitment. This is reflected in the result shown in Fig. 4B which demonstrates that the rate of CK accumulation was slower when the rescue by follistatin was performed at later times. Thus, these results clearly demonstrate that the effect of activin A is reversible and that the mode of action resembles that of TGF-β. As long as fresh TGF-β-containing medium is provided, inhibition of differentiation can be sustained, but reversal of the inhibition is observed after removal of TGF-β (Florini and Magri, 1989). The result shown in Fig. 4B raises the possibility that levels of CK accumulation of the all cultures released from activin A inhibition might reach nearly to that of the control culture if cultured for longer period. This result again strongly suggests that activin A inhibits the early phase of differentiation around commitment stage. It may be that the cells were arrested at commitment phase by activin A, but follistatin released them from the arrest by inactivating activin bound to its cell surface receptor. Olson *et al.* (1986) reported that mouse C2 cells become postmitotic in the presence of TGF-β. Myogenesis-inhibiting activity of TGF-β was not reversed by follistatin (data not shown). At present, our data show that the difference in the effect on myogenic differentiation between activin and TGF-β is solely the sensitivity to follistatin. Regarding the effect of follistatin on myogenic differentiation, our unpublished data suggest that follistatin exhibits a myogenesis-promoting activity at unphysiologically high concentrations and also influences at the phase of myotube growth. It is of much interest whether follistatin acts on myogenic cells directly or indirectly, possibly by inactivating activin secreted by cells themselves.

We also demonstrated that activin A inhibited the expression of MyoD1 which is known to participate in the

regulation of myogenic differentiation (Fig. 5). Inhibitions of MyoD1 expression have also been demonstrated for TGF-β1 (unpublished data). We are now investigating whether expression of other myogenesis regulating factors are affected by activin A.

Some bone morphogenetic proteins have also been reported to inhibit myogenic differentiation (Inada *et al.*, 1996). Therefore, it may be that members of TGF-β superfamily in general have the ability to inhibit in myogenic differentiation. However, the true nature of the action of activin and other members of TGF-β superfamily on myogenic differentiation remained to be resolved.

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