Follistatin Is a Developmentally Regulated Cytokine in Neural Differentiation*

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Activin acts mitogenically on P19 cells as well as being inhibitory of the differentiation of retinoic acid-treated P19 cells and some neuroblastoma cell lines. Here, we show some lines of evidence that follistatin, an activin-binding protein, is also involved in neural differentiation. Counteracting the activity of activin, addition of follistatin suppresses the anchorage-independent growth of P19 cells in soft agar and stimulates neurite outgrowth of a neuroblastoma cell line, IMR-32 cells. While activin does not seem to be expressed significantly, follistatin is demonstrated in the conditioned medium of these cells. Furthermore, the expression of follistatin in P19 cells is subject to dynamic fluctuations in response to retinoic acid treatment. These neural cells may produce follistatin in a cell stage-specific manner in order to interact with exogenously derived activin.

Activin, a member of the transforming growth factor-β family peptides, was first characterized from gonadal fluids as a stimulator of follicle-stimulating hormone (FSH) release from anterior pituitary gland (1, 2). Since erythroid differentiation factor was shown to be identical to activin-A (3, 4), diverse biological roles of activin outside the reproductive system have been extensively studied. The most intriguing finding may be the role of activin in embryogenesis. In amphibian systems, activin is supposed to be responsible for mesodermal induction (5-7). Activin induces different regions of drosophila tissue in a dose-dependent manner as well as anterior structures in axial pattern formation (8, 9). Thus, activin is involved in morphogenesis of early development. It should also be noted that activin acts distinctly from RA in various aspects of cell differentiation. In amphibian development, activin and RA differentially activate separate homeo boxes (10). In cultured mammalian cells, activin inhibits RA-induced differentiation of P19 cells and some types of neuroblastoma cells (11, 12). Activin can also inhibit the RA-induced secretion of alkaline phosphatase from osteoblastic cells. These observations could be explained by the presence of a RA-regulated mediator which interacts with activin.

The follistatin, a protein that can bind with activin and inhibit its activity (13, 14) appears a good candidate for it. Recent studies demonstrating follistatin transcripts in various tissues (15-18), such as kidney or brain, may indicate that follistatin has a significant role in extragonadal tissues. Here, we report some observations which suggest that follistatin is developmentally regulated to interact with activin in neural differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—P19 cells were obtained from Dr. M. W. McBurney (University of Ottawa). P19 cells were obtained from the Japanese Cancer Research Resources Bank (20). They were usually maintained in α modification of Eagle’s minimum essential medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories) and 10 μg/ml gentamycin sulfate (Shering-Plough) at 37 °C in a humidified atmosphere of 5% CO2.

Reagents—Recombinant activin A was produced using Chinese hamster ovary cells as previously described (4). Follistatin was purified from porcine ovaries as previously described (13).

Follistatin Treatment of IMR-32 Cells—IMR-32 cells (106/ml) were plated on 35-mm dishes in 2 ml of α-medium containing 5% FBS. After 24 h, cells were added with 0.3 μM RA and increasing amounts of follistatin (0, 0.05, 0.3, 3, 30 nM), 0.3 μM RA, and 1 nM inhibin, 0.3 μM RA, and 3 nM follistatin for the first 2 days and 1 nM activin for the last 3 days or no agents. After 5 days, phase-contrast photomicrographs were taken, and the cell number was counted by hemocytometer. In another experiment, cells were treated with follistatin (0, 0.05, 0.3, 3, or 30 nM) alone for 14 days. In this case, medium was changed at day 7 when cells were detached with Ca2+-free phosphate-buffered saline and replated. The concentration of follistatin is expressed in molar units by calculating the molecular mass of follistatin as 35 kDa (21). Cell numbers were statistically analyzed by Duncan's multiple range test.

Colony Formation Assay in Soft Agar—Anchorage-independent cell growth was measured in soft agar in 96-well plates as previously described (3) with some modifications. First, a 50-μl basal layer consisting of α-medium, 10% FBS, and 0.67% agar was prepared. Next, the middle layer containing 100/well of single cells in 50 μl of α-medium, 10% FBS, and 0.3% agar was overlaid. The top layer consisted of 100 μl of α-medium containing porcine follistatin at the indicated concentrations. Colonies were scored at day 9 after plating by counting aggregates whose diameter was more than 125 μm. Phase-contrast photomicrographs were simultaneously taken for some samples.

Northern Blotting—P19 cells were treated with 0.3 μM RA as previously described (19). In brief, cells were cultured in bacterial media in the presence of 0.3 μM RA for the first 4 days with a medium change at day 2 and then transferred to tissue culture dishes. 10 μg of poly(A)-enriched RNA isolated from cells at the indicated times was electrophoresed on agarose gel (1.0%) under denaturing conditions and blotted to a nylon membrane. Mouse activin receptor cDNA probe was produced by reverse transcription-polymerase chain reaction according to the published sequence (22). The sense primer, 5'-GCAAGGGGAAGATTTGGTTGTGTC-3' (the 666-880 oligonucleotide position of mouse activin receptor) was combined with the antisense primer 5'-CAATTCCTCATAGGACTAATCCCAT-3' (the
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FIG. 1. A, stimulatory effect of follistatin on the differentiation of IMR-32 cells. Cells were treated with 0.3 μM RA (a), 0.3 μM RA + 3 nM follistatin (b) for 6 days. Cells were also treated with (c) or without (d) 3 nM follistatin for 14 days. Phase-contrast photomicrograph was taken for each sample (magnification: a and b, ×132; c and d, ×265). B, effect of follistatin on the growth of IMR-32 cells as measured by cell count. a, cells treated with various agents in the presence of 0.3 μM RA for 6 days. Lanes 1–5, 0, 0.03, 0.3, 3, 30 nM follistatin, respectively; lane 6, 1 nM inhibin; lane 7, 3 nM follistatin for the first 2 days and 1 nM activin for the last 3 days; lane 8, no agents. b, cells treated with follistatin alone for 14 days. Lanes 9–13, 0, 0.03, 0.3, 3, 30 nM, respectively. Data are the means of four samples ± S.E. FOL, follistatin. *, p < 0.05 versus control lane 1; **, p < 0.01 versus control lane 9.

Among various neural cell lines investigated (IMR-32, GOTO, NB-1, SK-N-SH, and PC12), IMR-32 cells were found to differentiate in response to porcine follistatin. When cells were treated with 0.3 μM RA alone, the degree of morphological differentiation was not so striking (Fig. 1A, a). However, addition of more than 3 nM follistatin markedly stimulated the cellular aggregation and neurite outgrowth (Fig. 1A, b).
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**Fig. 2.** Suppressive effect of follistatin on the growth of P19 cells. Follistatin dose dependently suppresses the rates of colony formation of P19 cells in a soft agar (a). Phase-contrast photomicrograph was taken for samples with (b) or without (c) addition of 1 μM follistatin (magnification, × 32).

**Fig. 3.** Time course of follistatin and activin receptor mRNAs in RA-treated P19 cells. 10 μg of poly(A)+ RNA was isolated from cells treated with 0.3 μM RA at the indicated times. They were electrophoresed on 1.0% agarose gel under denaturing conditions, blotted to nylon membrane, and hybridized with mouse follistatin, mouse activin receptor, and β-actin cDNA probes.

The effect was completely and reversibly abolished by the addition of an equimolar activin (not shown). Follistatin, by itself, also stimulated the neural differentiation in a longer-term culture (Fig. 1A, c and d). Although neural differentiation markers for IMR-32 cells were not available, the differentiation-promoting effect was clearly reflected on the cell number. Follistatin significantly inhibited the growth of the cells in a dose-dependent manner either under the presence or absence of RA (Fig. 1B, a and b). Inhibin, by contrast, had no effects on the growth and differentiation of IMR-32 cells (Fig. 1B, a).

**Follistatin Suppresses the Growth of P19 Cells in a Soft Agar Assay**—Our preliminary results showed that follistatin had little effect on the RA-induced neural differentiation of P19 cells. However, follistatin dose dependently suppressed the anchorage-independent growth of P19 cells in soft agar, though the effective concentration was much higher than that employed on the differentiation of IMR-32 cells (Fig. 1B, a).

**The Expression of Follistatin and Activin Receptor mRNAs in P19 Cells**—Northern blotting showed an oscillating change of follistatin mRNA during the course of P19 differentiation (Fig. 3). The early response to RA was so rapid, which may suggest that the follistatin gene is a RA-regulated gene. After a transient peak of 24 h, the mRNA disappeared within 48 h and became significant again in the later stage. This characteristic pattern was reproducible. By contrast, the activin receptor mRNA was increased not rapidly but gradually at a later stage.

**The Production of Follistatin in the Conditioned Medium of**
P19 and IMR-32 Cells—Next, we demonstrated the presence of follistatin at the protein level. Fig. 4a shows the existence of molecules immunoreactive to follistatin in the medium of undifferentiated P19 cells and a drastic decrease after treatment with RA. Two forms of follistatin (32 and 35 kDa) are known to be produced by alternative splicing (21), and they are further modified with glycosylation (13, 25). The pattern of bands derived from the conditioned medium of P19 cells is similar to that of pituitary, where the 35-kDa band is more predominant (14). Ligand blotting using 125I-activin also showed a consistent result, although the sensitivity of detection was much higher than immunoblotting (Fig. 2b). Immunoblotting using a polyclonal antibody which recognizes the common peptide sequence between human and mouse follistatin could not detect the band in IMR-32 cells (data not shown). However, ligand blotting by long-exposure autoradiography showed that the follistatin band in IMR-32 cells was stronger than that of the control serum (Fig. 4c). Thus, the expression of follistatin in IMR-32 cells seemed to be much lower than P19 cells.

**DISCUSSION**

We and others have observed a series of activin effects on neural cells. Activin acts as a potent mitogen on P19 cells in an extracellular matrix-dependent manner (11, 26). Activin is also inhibitory on the neural differentiation of RA-treated P19 cells and some neuroblastoma cell lines (11, 12). Furthermore, activin acts as a survival factor for other types of neuronal cells (27). These observations suggest that activin plays significant roles in the nervous system. In pituitary, the secretion of FSH is controlled by activin and its related proteins. The FSH stimulatory effect of activin is neutralized by follistatin and activin acts as a survival factor for other types of neural cells. Activin acts as a potent mitogen on P19 cells in a proliferating state.

Follistatin exogenously added to IMR-32 cells may have further modified with glycosylation (13, 25). The pattern of bands derived from the conditioned medium of P19 cells seemed to be in line with the observations in gonadal tissues. It has been confirmed that granulosa cells in ovary produce follistatin (28), whereas the origin of activin is still obscure. It is intriguing if a similar mechanism of differentiation is employed in these two different systems.

The physiological role of follistatin also remains to be disclosed. Recently, follistatin was characterized as a heparin binding protein, suggesting that it regulates activin action by binding protein, suggesting that it regulates activin action by a heparin binding property. Activin and activin receptor which interact with the exogenously derived activin in a developmentally regulated manner, seem to be in line with the observations in gonadal tissues. It has been confirmed that granulosa cells in ovary produce follistatin (28), whereas the origin of activin is still obscure. It is intriguing if a similar mechanism of differentiation is employed in these two different systems.

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