The Stimulatory Effect of Activin on PRL Production in GH3 Cells in a Serum-Free Culture

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Background and Aims : Activin was originally found as a stimulating factor of follicle stimulating hormone in pituitary cell cultures.   To establish a simple culture system for studying the effects of activin on prolactin production, we examined the effects of activin in rat somatolactotroph derived GH3 cells cultured in a serum free condition.   **Methods :** GH3 cells were cultured in Ham's F10 medium containing 2 mM L-glutamine and 0.1% (w/v) BSA with activin, follistatin (an activin binding protein), or a vehicle (control).   **Results :** The cell viability was between 80 to 90% throughout the 96 hour incubation period in the three groups.   Activin stimulated prolactin production in a time dependent (24-96 hours) and dose dependent (75-300 ng/ml) manner.   A co-treatment with graded doses of follistatin and 100 ng/ml of activin suppressed the increase of prolactin production brought about by activin in a dose dependent manner, and the production decreased to the control level after the addition of 300 ng/ml of follistatin.   **Conclusions :** The present study demonstrated the stimulatory effect of activin on prolactin production and the antagonism between activin and follistatin in GH3 cells in a serum free condition. This culture system is thus useful to study the effects of activin on prolactin production. (Kitakanto Med J 2006 ; 56 : 1～9)

Key Words : Activin, Follistatin, Prolactin, GH3 cells

Introduction

Activin, initially isolated from the gonads based on its ability to stimulate the secretion of follicle stimulating hormone (FSH),[1,2] is a member of the transforming growth factor ß (TGF-ß) superfamily.[3]   It is produced in a broad range of tissues, including the anterior pituitary gland,[4] and has a wide variety of biological actions ; it is a key regulator of the autocrine and paracrine functions of the pituitary gland.[5,6]

Activin stimulates FSH synthesis by inducing the expression of the FSH beta subunit mRNA without changing the expression level of the luteinizing hormone (LH) beta subunit.[7]   On the other hand, activin have also been reported to have suppressive effects on the secretion and production of other pituitary hormones.   Activin was shown to suppress the secretion of adrenocorticotropic hormone in the rat corticotroph derived AtT20 cells.[8]   Somatotrophs and lactotrophs have also been shown to be target cells for the actions of activin.[9]   Both the basal and stimulated secretion of

growth hormone (GH) and PRL were found to be suppressed by activin in primary rat anterior pituitary cell cultures.[10~12]   In primary pituitary cell cultures, the number of PRL producing cells decreased in the presence of activin.[13]   Activin also suppressed PRL synthesis in GH3 cells, a rat pituitary cell line, cultured in a serum containing medium.[14]   Activin was shown to have inhibitory effects on both cellular proliferation and PRL synthesis in another pituitary cell line, GH4C1.[15,16]   Activin reportedly suppressed cell growth and Pit-1 and PRL gene expression through Smad2 and menin in GH4C1 cells.[16]   It was reported that activin decreased GH expression through multilevel regulation of the transcription factor Pit-1 in MtTW15 cells, derived from a rat pituitary somatotroph tumor.[17,18]

On the other hand, activin has also been shown to have stimulatory effects on GH production in GH3 cells.[14]   GH3 cells are a well-established cell line derived from a rat anterior pituitary tumor,[19] that produce both GH and PRL.[20]   They are widely used

to investigate the actions of polypeptides[21] and have also been shown to express both type I and type II membrane receptors for activin.[22,23]

Cross talk has been reported between activin signalling and other signalling pathways of growth factors, many of which are contained in the serum.[24,25] Follistatin, found in the serum, has been shown to modify the effects of activin.[5,26] Therefore, it is not easy to conclusively determine whether the observed changes of PRL production in cells cultured in the presence of serum are attributable only to the effects of activin or are also a result of the modifying actions of other serum factors affecting activin signalling. Therefore, we used GH3 cells cultured in a "serum free" condition, a simple culture system, to investigate the effects of activin on PRL production. Cell proliferation assay was conducted by measurement of the extent of BrdU incorporation, and PRL production was determined by measurement of the amount in the culture of PRL protein, the end product of PRL gene expression.

Materials and methods

Reagents

Recombinant human activin A,[27] kindly provided by Dr. Yuzuru Eto, Ajinomoto Co., Inc (Kawasaki, Japan), was used for the experiments. Recombinant human follistatin-288[28] was used as follistatin, and the recombinant human follistatin-288 (Lot No. B4384) and rat PRL RIA kit were obtained through NHPP, NIDDK & Dr. A.F.Parlow (Bethesda, MD & Harbor-UCLA Med Ctr, Torrance, CA). GH3 cells (ATCC CCL-82.1) were purchased from Dainippon Co., (Oosaka, Japan). All the other chemicals used were of commercial grade.

Cell culture

GH_3 cells were maintained in Ham's F-10 medium supplemented with 2 mM L-glutamine, 15% horse serum and 2.5% FBS without antibiotics, for a maximum of 10 weekly passages.

On the day of the experiment, the cells were washed three times with Ham's F-10 medium and seeded in multi-well plates with 24 or 96 non-coated wells (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 2.5×10^4 cells/cm². Ham's F-10 medium supplemented with 2 mM L-glutamine and 0.1% (w/v) BSA without antibiotics was used in the experiment. Cells were cultured in a humidified atmosphere containing 5% CO_2-95% air, at 37C.

Cell Proliferation

Cell proliferation was measured using a hemocytometer, and by the 5-bromo-2'-deoxyuridine (BrdU) incorporation method.

Cells were plated at the density of 50,000 per 2 cm² well (25,000 cells/cm²) in 300 μl of serum free medium, in the presence of activin (100 ng/ml), follistatin (100 ng/ml), or a vehicle (control). After 24-96 hours of incubation, the cells were detached from the well by the addition of 10 μl ethylenediaminetetraacetic acid (0.1 M) for 10 min at 37C and resuspended in the well.[15] An aliquot of the cell suspensions was loaded on to a hemocytometer with trypan blue dye (1 : 1, vol/vol), the cell number was counted, and the cell viability was determined by dye exclusion.

The cell proliferation activity was also determined using a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. The BrdU enzyme-linked immunosorbent assay was performed in accordance with the manufacturer's instructions (Catalogue No.1-444-611, Roche Diagnostics, Mannheim, Germany). Briefly, cells were seeded into 96-well multititer plates at a density of 8,000 per 0.32 cm² well (25,000 cells/cm²) in 100 μl of serum free medium, in the presence or absence of activin or follistatin. After incubation for the indicated times, 10 μl of 100 μM BrdU solution was added to each well, and the cells were reincubated for another 2 hours. The culture medium was removed, the cells were fixed, and the DNA was denatured. The cells were then incubated with mouse anti-BrdU monoclonal antibody conjugated to peroxidase at room temperature for 90 minutes. After removal of the antibody, the immune complexes were detected by subsequent reaction with tetramethylbenzidine. The reaction product was quantified by measuring the absorbance at 370 nm with a reference wavelength of 492 nm in Wallac 1420 ARVOsx (Wallac, Turku, Finland). The extent of BrdU incorporation into the cells in each well of the 96-well multititer plate was assessed by the absorbance in each well after subtraction of the average absorbance in four blank wells.

Hormone Assay

Cells were plated at a density of 8,000 per 0.32 cm² well of a 96 multi-well plate (25,000 cells/cm²) in 200 μl of serum free medium, with or without activin or follistatin. After a predetermined incubation period, the culture medium was removed and the cells were lysed by incubation with 0.1% (w/v) Triton X-100, 0.5% BSA, PBS, followed by freezing and thawing as previously described.[29] The PRL concentrations in the medium and the cell lysate were measured with the rat PRL RIA kit, using rPRL RP-3 as the standard. The intra- and interassay coefficients of variation were 10.4% and 11.4%, respectively.

### Effect of activin on PRL production vs. on cell proliferation

The ratio of PRL production per BrdU incorporation after 72 hours of culture was calculated using the data from four independent experiments. GH3 cells were cultured in Ham's F10 medium containing 2 mM L-glutamine and 0.1% (w/v) BSA in the presence of activin (100 ng/ml), follistatin (100 ng/ml), or a vehicle (control), for 72 hours. The assays in each experiment were conducted in quadruplicate. PRL production, calculated as the sum of the amount of PRL in the medium and in the cell lysate in each well, was divided by the value of BrdU incorporation, as determined based on the absorbance, in the corresponding well.

Statistical analysis

The data from quadruplicate cultures are presented as the mean±SE. Comparisons between groups were performed using one-way ANOVA. The significance of the differences between the mean values in the control group and in each treated group was tested using Fisher's PLSD test. $P < 0.05$ was considered to be statistically significant.

Results

Previous studies had shown that activin decreased the number of PRL producing cells in pituitary cell cultures and the cell proliferation activity in GH4C1 cells. Therefore, first we characterized the effects of activin on the cell proliferation activity in GH3 cells cultured in our serum free condition, by trypan blue dye exclusion with hemocytometry and measurement of BrdU incorporation. Determination of the total cell number and the viable cell number in activin (100 ng/ml)-, follistatin (100 ng/ml)-, or vehicle-treated cell cultures revealed a time dependent increase of the two parameters over a 24 to 96 hour period. Although the total cell number in the activin treated cell cultures were 142% and 136% of that in the control after 72 hours and 96 hours, the difference was not significant at either time point. The cell viability was between 80 to 90% throughout the 96 hour incubation period, and there were no statistically significant differences among the three groups. The time course of changes in the cell proliferation activity, determined based on the extent of BrdU incorporation, were measured in the GH3 cells cultured in the presence of activin (100 ng/ml) or a vehicle after 96 hours of incubation. The absorbance at 370 nm and the relative absorbance of the activin treated cells to that of the control at each indicated time point are shown in Figure 1. Activin stimulated BrdU incorporation in the GH3 cells. After 72 hours, significantly greater BrdU incorporation (156% relative to that in the
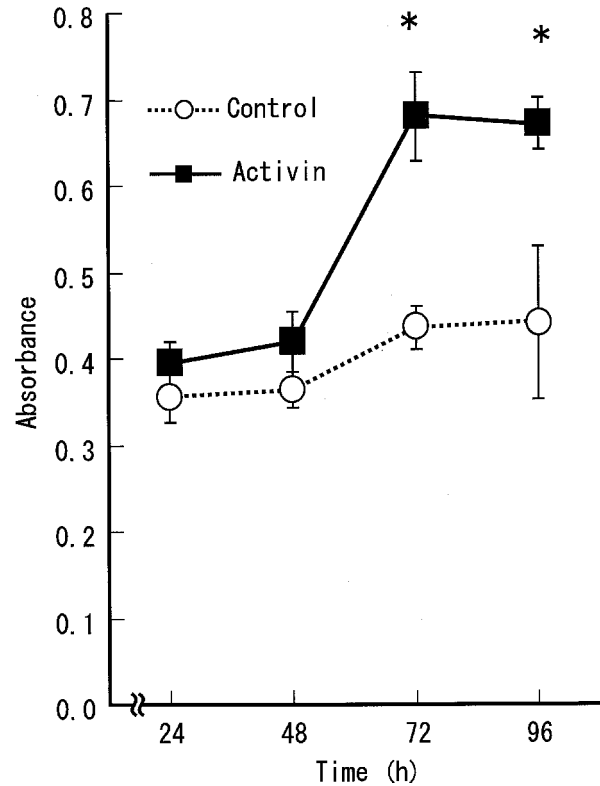

Fig.1 Effects of activin on cell proliferation in GH3 cells cultured in the absence of serum over time. GH3 cells were plated at a density of 8,000 cells per 0.32 cm² well in 100 μl of Ham's F10 containing 2 mM L-glutamine and 0.1% (w/v) BSA, in the presence of activin (100 ng/ml) or vehicle (control). At the indicated time points (24, 48, 72, and 96 hours), 10 μl of 100 μM BrdU solution was added to each well, as described in Materials and Methods. In each experiment, the absorbance at 370 nm was measured after 20 minutes of substrate reaction. The filled rectangles represent activin and the clear circles represent the control. The results from the assays conducted in quadruplicate are shown as mean ± SE (n = 4). *, P < 0.01 as compared with the value in the control at each time point.

vehicle treated control) was noted in the activin treated cells than in the control. BrdU incorporation in the cells cultured in the presence of 100 or 300 ng/ml activin was also estimated after 72 hours of incubation, and a dose dependent increase in the BrdU incorporation was observed (Fig. 2).

Next, we examined the effect of activin on the PRL production in the GH3 cells. Cells were incubated with activin (100 ng/ml), follistatin (100 ng/ml), or a vehicle (control). Both the basal secretion and the total PRL production increased over time in all the three groups (Fig. 3). Furthermore, the addition of activin accelerated the time dependent increase of PRL production. The PRL production was significantly higher after 72 hours of incubation in the cells cultured in the presence of activin. The PRL production in the cells cultured in the presence of activin was 175% and 249% relative to that in the control after 72 hours and
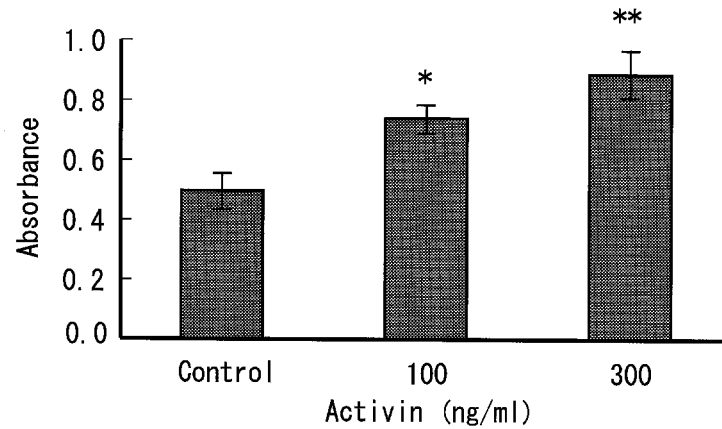
Fig.2   Dose-related effect of activin on cell proliferation in GH3 cells cultured in the absence of serum. GH3 cells at a density of 8,000 per 0.32 cm² well in 100 μl of Ham's F10 containing 2 mM L -glutamine and 0.1% (w/v) BSA were incubated with 100 or 300 ng/ml activin for 72 hours, and the BrdU incorporation was measured as described in Materials and Methods.   The absorbance at 370 nm is shown as mean $\pm$ SE (n = 4). $*$, P<0.05 as compared with the control value. $**$, P<0.01 as compared with the control value.
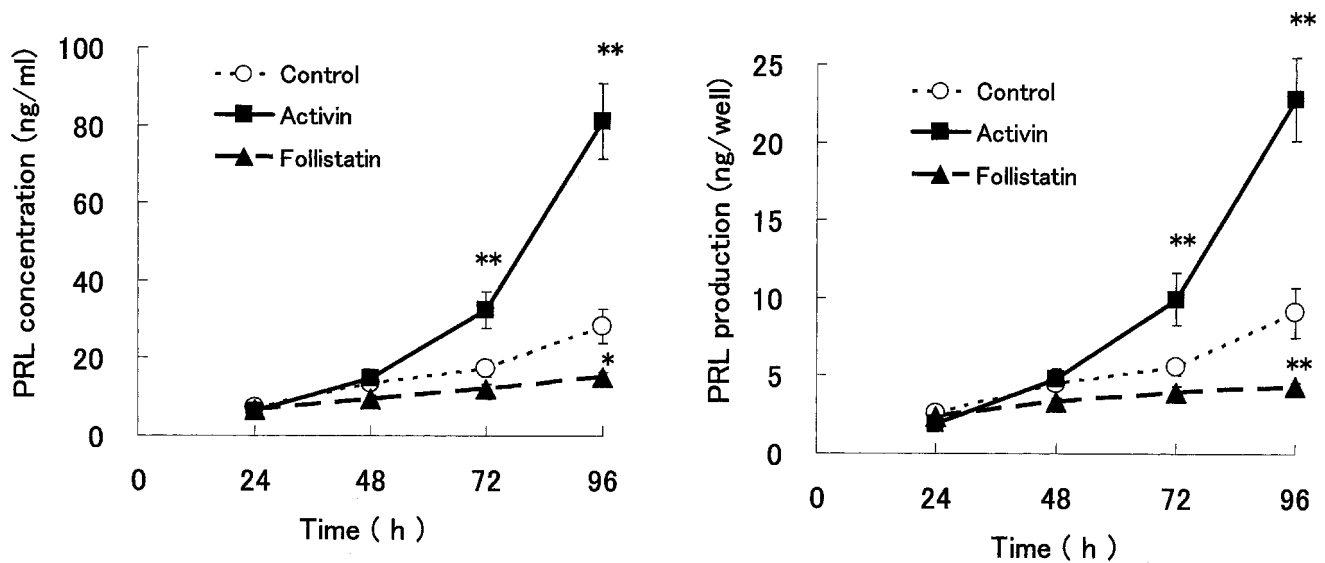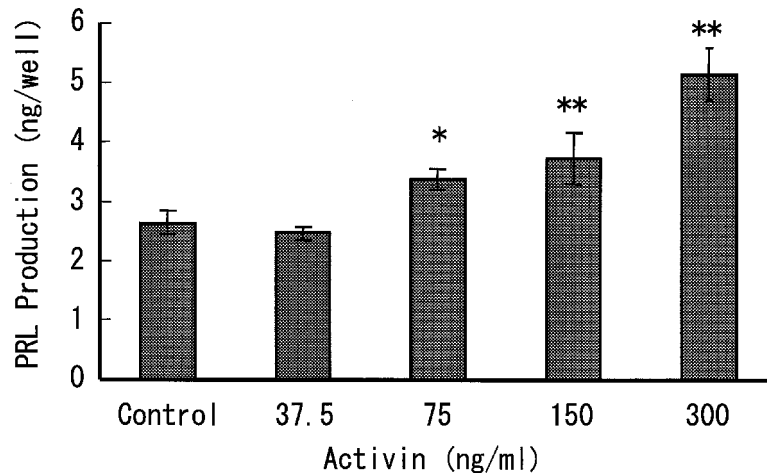

Fig.3   Effects of activin and follistatin on PRL production in GH3 cells cultured in serum free medium over time.   GH3 cells at a density of 8000 cells per 0.32 cm² well were cultured in Ham's F10 containing 2 mM L-glutamine and 0.1% (w/v) BSA, in the presence of activin (100 ng/ml), follistatin (100 ng/ml), or vehicle (control).   At each of the indicated time points, the culture medium was removed, the cells were lysed, and the amount of PRL in the medium and cell lysate were measured, as described in Materials and Methods.   Closed rectangles : activin.   Closed triangles : follistatin.   Open circles : control.   The result from the assays conducted in quadruplicate are shown as mean $\pm$ SE (n = 4). $*$, P<0.05 as compared with the control value at each time point. $**$, P<0.01 as compared with the control value at each time point.   Left panel : PRL concentrations in the medium.   Right panel : PRL production per well, calculated as the sum of the amount of PRL in the medium and in the cell lysate.

96 hours of incubation.   Follistatin, on the other hand, suppressed the time dependent increase of PRL production, although no significant difference was observed until 96 hours of incubation.

To examine the dose dependent effect of activin on PRL production, GH3 cells were cultured in a serum free medium in the presence of various concentrations of activin, for 72 hours.   Activin was found to stimulate PRL production in a dose dependent manner ; the PRL production in the presence of 300 ng/ml was

194% of that in the control (Fig. 4).   However, no significant effect of follistatin, at concentrations between 37.5 to 300 ng/ml was observed on PRL production after 72 hours' incubation.

To assess whether the increase in hormone production observed in the presence of activin was a consequence of the increase in the cell proliferation activity, the effect on the PRL production vs.   on cell proliferation was analyzed after 72 hours of culture.   The PRL production per unit BrdU incorporation in the cells

Fig.4 Dose-related effects of activin on PRL production in GH3 cells cultured in serum-free medium. GH3 cells at a density of 8,000 per 0.32 cm² well were cultured in Ham's F10 medium containing 2 mM L-glutamine and 0.1% (w/v) BSA in the presence of various concentrations of activin, for 72 hours. The results are shown as the mean $\pm$ SE (n = 4). $*$, P<0.05 as compared with the control (activin ; 0 ng/ml) value. $**$, P<0.01 as compared with the control value.
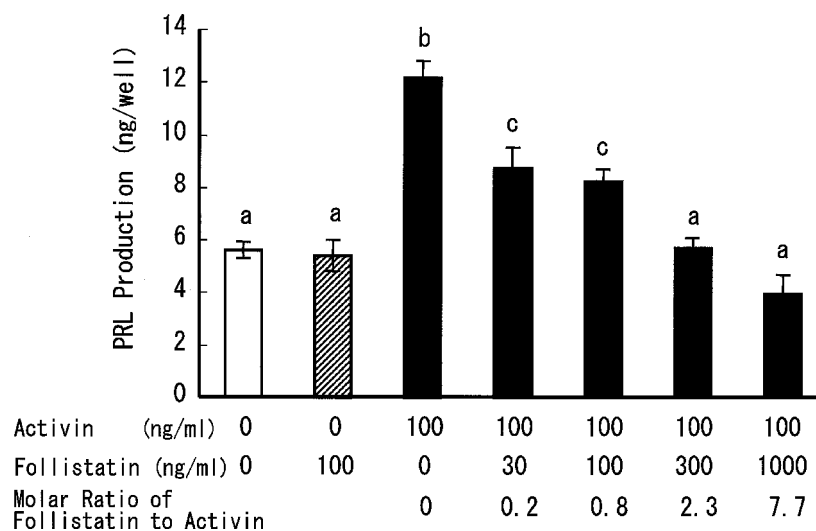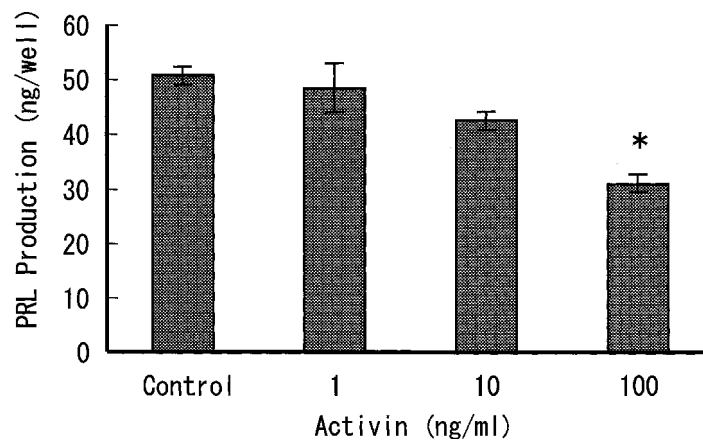

Fig.5 Dose-related effects of follistatin on PRL production in GH3 cells cultured in serum-free medium in the presence of 100 ng/ml activin. GH3 cells at a density of 8,000 per 0.32 cm² well were cultured in Ham's F10 medium containing 2 mM L-glutamine, 0.1% (w/v) BSA and 100 ng/ml activin in the presence of various concentrations of follistatin, for 72 hours. Open bars (control) : PRL production of in GH3 cells cultured in the presence of neither activin nor follistatin. Diagonal bars : PRL production in GH3 cells cultured in the presence of 100 ng/ml follistatin but no activin. Closed bars : PRL production in GH3 cells cultured in the presence of 100 ng/ml activin and follistatin at various concentrations of follistatin. The results are shown as the mean $\pm$ SE (n = 4). Bars with different letters (a-c) denote significant differences (P<0.05).

cultured in the presence of activin (100 ng/ml) was significantly higher than that in the control (236 $\pm$ 2.3% of that in the control ; P<0.01), indicating that the effect of activin on PRL production was more pronounced than that on the cell proliferation activity. However, there was no significant difference in this parameter between the cells cultured in the presence of follistatin (100 ng/ml) and the control cells.

Follistatin is an activin binding protein known to antagonize the biological activity of activin. To assess this antagonistic effect of follistatin on activin, GH3 cells were cultured in the presence of various concentrations of follistatin plus 100 ng/ml of activin, for 72 hours. The PRL production increased significantly in the cells cultured in the presence of activin, however, co-treatment with follistatin suppressed this activin-induced increase in PRL production, in a dose dependent manner. The PRL production was suppressed to the level observed in the control in the presence of 300 ng/ml follistatin in cells treated with

Fig.6   Effect of activin on the PRL production in GH3 cells cultured in serum-supplemented medium. GH3 cells at a density of 8,000 per 0.32 cm² well were cultured in Ham's F10 medium containing 15% horse serum and 2.5% FBS with various concentrations of activin for 72 hours, and the amount of PRL produced was measured.   The results are shown as the mean ± SE (n = 4).   *, P<0.01 as compared with the control value.

100 ng/ml of activin (Fig. 5).   Thus, in the presence of a molar ratio of follistatin : activin of greater than 2 : 1, the stimulatory effect of activin on PRL production was antagonized by follistatin.

To determine whether the stimulatory effect of activin on PRL production was affected by the presence of serum, we added 15% horse serum and 2.5% FBS instead of 0.1% BSA to the medium, and the GH3 cells were cultured for 72 hours.   The results revealed that PRL production was suppressed by activin in the presence of serum (Fig. 6).

Discussion

Activin has been shown to have antiproliferative effects against many types of cells, including PRL containing cells of the rat anterior pituitary[13] and other rat somatolactotroph derived cell lines, such as GH4C1.[15,16]   On the other hand, several types of cells have also reported to show enhanced proliferative activity in response to treatment with activin.[30~32]   In our serum free system, activin stimulated GH3 cell proliferation.   Kojima et al reported that activin inhibited DNA synthesis in Balb/c 3T3 mouse fibroblasts cultured in the presence of serum but stimulated the synthesis in a serum free medium.[30]   Activin was much less potent than platelet-derived growth factor in stimulating DNA synthesis, and they speculated that the stimulatory actions of activin were not detected in the presence of serum, since serum contained a significant amount of platelet-derived growth factor.

Opposite effects of a ligand on cell proliferation in the presence/absence of serum has also been reported in GH3 cells.   Epidermal growth factor[33] inhibited the proliferation of GH3 cells in a serum supplemented medium, whereas a stimulatory effect was seen in a

serum free medium.

It is noteworthy that most, if not all, reports of the stimulatory effects of activin on cell proliferation are based on studies using cultures performed under a serum free condition.[30~32]   On the other hand, many reports describing the inhibitory effects of activin used serum supplemented medium for the cell culture.[13,15,30] Serum contains growth factors that stimulate cell cycle progression, therefore, it may be difficult to detect the stimulatory effect of activin on cell proliferation in the presence of serum.

Activin is produced in the pituitary gland and act as an autocrine/paracrine factor.   Although there is not such a definitive report supporting the physiological role of activin on the production and secretion of PRL, as on those on FSH, it is reported that activin inhibited the production and secretion of PRL in a primary pituitary cell culture using immature female rat pituitary cells.[12]   It is also reported that activin decreased the percentage of PRL secreting cells and mean PRL secretion per cell in a primary pituitary cell culture using mature female rat pituitary cells.[13]   In our study, activin stimulated PRL production in GH3 cells cultured in a serum free medium, and this is the first report of the stimulatory effect of activin on PRL production.   The anterior pituitary is composed of a complex network of endocrine and non-endocrine cells, which affects PRL production.   It may be easier to detect the stimulatory effect of activin on PRL production in a single cell population such as GH3 cells, which does not possess the plural components in the anterior pituitary gland.

The effect of activin on PRL production switched from stimulatory to inhibitory with the addition of serum to the culture medium in our system.   The

inhibitory effect on PRL production in GH3 cells cultured in a serum supplemented medium is consistent with a previous report using GH3 cells cultured in a serum supplemented medium.[14] In GH4C1 cells, activin suppressed PRL production both in a serum containing medium[15] and in a serum free or low serum (2% FBS) condition as shown in the previous reports.[16] Even though both the cell lines are closely related, opposite effects of a ligand on the PRL production have been reported between GH3 and GH4C1 cells[34]; Insulin-like growth factor-1 decreased PRL mRNA expression in GH3 cells, while it increased PRL mRNA expression in the GH4C1 cells. The opposite effect of activin on PRL production reported between our study and previous reports[15,16] could be attributable to the difference in the cell types used for the study.

Follistatin is produced in a number of diverse tissues, including gonadotrophs, somatotrophs and folliculostellate cells in the anterior pituitary gland, and has been presumed to control the effects of activin in the anterior pituitary cells in a paracrine or autocrine manner.[5,6] Although follistatin alone showed no significant effect on PRL production even at doses up to 300 ng/ml until 72 hours of culture, a decrease in PRL production was noted after 96 hours of incubation. Expression of the inhibin/activin βA subunit mRNA and protein has been reported in GH3 cells.[22] Follistatin may play a role in decreasing PRL production by neutralizing the endogenous activin.

The actions of follistatin are mostly attributed to their ability to form a complex with activin and biologically antagonize its effects.[26] A ternary complex is presumed to be composed of two follistatin molecules bound to one activin molecule via its two ß subunits.[35] When graded doses of follistatin were added together with 100 ng/ml of activin to cell cultures, a dose dependent effect was seen. A reversal of the effects of activin on PRL production occurred after 72 hours of culture, when the follistatin : activin molar ratio exceeded 2 : 1. It has been reported in several *in vitro* systems that co-incubation with a constant amount of activin but increasing concentrations of follistatin prevents the biological activities of activin when the follistatin : activin molar ratio is greater than or equal to 2 : 1.[36] Our present observations show antagonism between the actionss of follistatin and activin on PRL production for the first time. However, this result was consistent with previous reports on other types of cells concerning the negative regulation of other biological effects of activin, such as the stimulation of FSH production, by follistatin.[5,6]

Insulin has been shown to stimulate PRL promoter activity in GH3 cells.[37] Insulin is contained in serum and is often used a supplement in serum free cell cultures. Since our culture system was simply composed of Ham's F10 and BSA without insulin, the basal PRL production was lower in our system as compared to that in media containing serum, and the effect of activin on PRL production was switched from stimulatory to inhibitory by the addition of serum. Although the mechanism involved in this switch remains clarified, it appears that our system is useful for investigating the effects of ligands in the serum that affect activin signalling on PRL production.

In conclusion, using GH3 cells cultured in a serum free medium, we showed that activin has stimulatory effects on cell proliferation and PRL production, and that follistatin antagonizes the stimulatory effect of activin on PRL production. The addition of serum caused a switch of the effect of activin on the cellular PRL production from stimulatory to inhibitory. Our culture system is thus useful for further investigation of the effects of activin on the cellular PRL production.

Acknowledgements

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