Paracrine Effect of Folliculo-Stellate Cells on the Growth Factor-Like Action of Activin A in Anterior Pituitary Cultures

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Abstract. Several studies have shown that pituitary folliculo-stellate (FS) cells exhibit local functions within the pituitary gland. On the other hand, we have shown previously that activin A increases the number of FSH-producing gonadotropes in cultured rat anterior pituitary cells. In this study, we investigated whether FS cells exert an influence on the action of activin A. FS cells were prepared by culturing the dispersed rat anterior pituitary cells in media containing 15% fetal calf serum and 6 mM glutamine for 15 days. Most cells had the morphological characteristics of FS cells and S-100 protein immunoreactivity, a specific marker of FS cells. The number of FSH cells, which was higher in activin A-treated than in control cultures, was reduced to the control level by incubation with activin A plus conditioned media from FS cell-enriched cultures (FSCM). This inhibitory effect of FSCM was neutralized by a follistatin antibody, but not by anti-S-100 protein or anti-basic fibroblast growth factor. Furthermore, follistatin suppressed activin A stimulated increases in the number of FSH cells in a similar inhibitory pattern to that of FSCM. Meanwhile, the number of FSH cells was not affected by FSCM or follistatin in the absence of activin A. These results suggest that FS cells are involved in the regulation of the function and/or the morphogenesis of the FSH cell-lineage by affecting the action of activin A, and that this paracrine effect of FS cells is mediated by follistatin.

Key words: Folliculo-stellate cell, Activin, Follistatin, FSH-gonadotrope, Paracrine effect.

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ACTIVINS composed of two β_A -subunits (activin A) or of β_A - and β_B -subunits (activin AB) of inhibins, were originally isolated from ovarian follicular fluid as stimulators of FSH secretion [1, 2]. Subsequent studies have shown that activins, members of the transforming growth factor- β superfamily [3], can affect cell growth and differentiation in various *in vitro* settings: The activities identified to date include erythroid differentiation [4, 5], germ cell proliferation [6], folliculogenesis [7], nerve cell survival [8],

embryogenesis [9–11] or pituitary somatotrope growth [12].

Growth- or differentiation-related effects of activins have also been noted in our previous reports which show that activin A increases the number of FSH-producing gonadotropes in primary cultures of rat anterior pituitary cells [13, 14]. The physiological importance of these growth factor-like activities of activins has been supported by the finding that the messenger RNAs (mRNAs) of their subunits are expressed in various tissues [15]. In the pituitary gland, the expression of mRNAs encoding the α and β_B -subunits of activins/inhibins was demonstrated [15], and immunoreactivity of these subunits was localized in gonadotropes [16]. These observations raise the

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possibility that activins and inhibins may serve as paracrine and/or autocrine factors within the target organs [17].

We showed in a previous report that the stimulatory effect of activin A on FSH secretion and biosynthesis was cell density dependent. That is, activin A failed to stimulate FSH secretion and biosynthesis when cell density was relatively high [13]. This cell density dependency has also been reported concerning the effect of activin A on erythroid differentiation in murine erythroleukemia cells [18]. These results, together with the possibility of activins functioning as paracrine and/or autocrine factors, suggest that some cell-to-cell interactions affect the expression of their actions.

In the anterior pituitary gland, the existence of a population of agranular cells, folliculo-stellate (FS) cells, was described nearly 40 years ago [19]. Several functions of these cells have been inferred from their morphological characteristics: a supportive role, phagocytosis, regulator of the microcirculation in the interstitial cavities, and a metabolic role, or a stem cell function, etc. [for review see ref. 20]. In addition to these presumptive functions, several studies have shown that FS cells produce bioactive molecules [21–25] including follistatin [24], recently shown to be an activin-binding protein [26], and that some of these molecules affect the secretion of pituitary hormones [21, 27–29]. These observations suggest that FS cells may also exhibit local functions with these humoral factors within the pituitary gland.

On these grounds, the present study was undertaken to examine whether FS cells in the pituitary gland influence the effect of activin A on the number of FSH-producing gonadotropes. For this purpose, an FS cell-enriched population was prepared from rat anterior pituitary glands by the method of Ferrara *et al.* [30] with some modifications. Conditioned media from these cultures and activin A were used to treat primary cultures of rat anterior pituitary cells.

Materials and Methods

Animals

Male Wistar-Imamichi rats, obtained from Imamichi Institute for Animal Reproduction

(Ohmiya, Japan) at the age of 8 weeks, were used throughout this study. The animals were kept in a controlled environment at $23\pm1^{\circ}\text{C}$ with a 14-h light: 10-h dark cycle (lights on at 0500 h), with free access to water and standard laboratory chow.

Reagents

Recombinant human activin A (erythroid differentiation factor) [31] was a gift from the Central Research Laboratory of Ajinomoto Co. (Kawasaki, Japan). Follistatin (activin-binding protein) was purified from rat ovarian follicular fluid [26]. Other materials were purchased from the following sources: Dulbecco's modified Eagle medium (DMEM, 0.1% glucose), fetal calf serum (FCS), deoxyribonuclease (DNase), pancreatin and trypsin from Grand Island Biological Co. (Grand Island, NY, USA); HEPES, BSA (fatty acid-free), penicillin and streptomycin sulfate from Sigma Chemical Co. (St. Louis, MO, USA); collagenase (Type II) from Worthington Biochemical Co. (Freehold, NJ, USA); L-glutamine from Wako Pure Chemical Industries Co. (Osaka, Japan); EDTA from Dojindo Laboratories Co. (Kumamoto, Japan).

Antibodies

Anti-S-100 protein was raised by immunizing mice against bovine brain S-100 protein (Sigma Chemical Co., St. Louis, MO, USA). The titer of the antiserum was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [32]. The antibody recognized both S-100 proteins A and B (Sigma Chemical Co., St. Louis, MO, USA) with similar titers (data not shown). Anti-follistatin serum was generated in rabbits against a synthetic peptide of the inner portion (residues 123–134) of human follistatin combined with *Limulus polyphemus* hemolymph hemocyanin [32]. Anti-basic fibroblast growth factor (bFGF) serum was generated in rabbits against a synthetic peptide of the N-terminus portion (residues 1–10) of bovine bFGF combined with bovine thyroglobulin [33].

Cell dispersion of anterior pituitary cells

Anterior pituitary glands were minced and washed with HEPES buffered saline (137 mM

NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM HEPES, pH 7.3), and cell dispersion was performed by the method reported previously [13, 14]. Briefly, the tissue was dispersed by incubation first in HEPES buffered saline containing 0.4% collagenase, 0.4% BSA and 0.2% glucose, second in 10% pancreatin, and finally in 200 mg/ml DNase. Cell viability determined by trypan blue exclusion was more than 90%, and $1.2\text{--}1.8 \times 10^6$ viable cells were obtained per rat.

Culture of folliculo-stellate cells

Folliculo-stellate (FS) cell-enriched cultures were prepared by a modification of the method reported by Ferrara *et al.* [30]. Dispersed anterior pituitary cells from 15–20 week-old rats were suspended in DMEM supplemented with 15% FCS and 2 mM glutamine (total 6 mM glutamine) at a concentration of 3×10^5 cells/ml, and 5 ml of the cell suspension was added to each well of 6-well culture dishes (Costar, Cambridge, MA, USA). The cells were incubated under a saturated atmosphere of 5% CO_2 -95% air in a CO_2 incubator at 37°C and the medium was changed every 3–4 days. One or 2 days after reaching confluency, unattached cells and debris were washed off and the cultures were dissociated by exposure to 10 mM PBS (pH 7.4) containing 0.05% trypsin and 0.3% EDTA. After centrifugation, the pellets were resuspended in the medium and the cells were further cultured under the same conditions. After the second passage, some cultures were immunostained by the peroxidase-antiperoxidase (PAP) complex method [13, 14] against S-100 protein, a specific marker of FS cells in the anterior pituitary [34]. If FS cells had become dominant in the cultures, the medium was replaced by DMEM containing 0.1% BSA and incubated for 24 h. Then the medium was collected and centrifuged ($480 \times g$ for 6 min), and the supernate was stored at -80°C as the FS cell-conditioned medium (FSCM). For the control, conditioned medium was prepared in the same way from the fresh cultures after 24-h incubation from plating, and referred to as DICM (day 1 conditioned medium).

Primary culture of anterior pituitary cells and treatments with activin A, FSCM, follistatin and the antibodies

Dispersed anterior pituitary cells from 10–15

week-old rats were adjusted to a concentration of 1×10^5 cells/ml with DMEM containing 10% FCS, and 160 μl of the cell suspension was added to each well of 96-well culture dishes (Costar, Cambridge, MA, USA). The cells were incubated under a saturated atmosphere of 5% CO_2 -95% air in a CO_2 incubator at 37°C. One day later red blood cells, unattached cells and debris were washed off, and the cultures were further incubated for 3 days in fresh medium containing 10% FCS and experimental agents such as activin A (10 ng/ml), FSCM, follistatin or the antibodies.

Immunostaining of FSH cells

After washing three times with 10 mM PBS (pH 7.6), the cells were fixed in Bouin's fluid (without acetic acid) for 10 min at room temperature and then stained immunocytochemically with streptavidin-biotin (SAB) reagents (Nichirei Co., Tokyo, Japan). Immunostaining was carried out according to the procedure described in the explanatory note accompanying the kit. Briefly, the sequences and reaction times were as follows: 3% hydrogen peroxidase for 10 min; normal goat serum for 10 min; primary antibody (1:1,250, NIDDK-anti-rFSH-S-11) for 1 h; biotin-labelled goat anti-rabbit IgG for 10 min; horse radish peroxidase-labelled streptavidin for 5 min; and peroxidase substrate (3-amino-9-ethylcarbazole) for 2 min. All steps were run at room temperature. In some wells, normal rabbit serum was used instead of the primary antibody in order to evaluate non-specific stainings. More than 200 cells from five random areas in each well were counted under a light microscope, and visibly stained cells were considered immunoreactive FSH cells.

Statistical analysis

Each value is the mean $\pm$ SEM for triplicate wells from a representative experiment. Reproducibility was verified by two or more independent experiments as noted in each legend. The data were statistically evaluated by Duncan's new multiple-range test, and differences of $P < 0.05$ were considered significant.

Results

Folliculo-stellate cell-enriched cultures

After 1 day in culture, most of the clumps and single cells were attached to the surface, while red blood cells, debris and unattached cells were

Fig. 1. Light micrographs of immunostained cells (red color) using either antibody to S-100 protein (a and b) or non-immune mouse serum (c). There were very few cells with S-100 protein immunoreactivity after 1 day in culture (a). After 15 days in culture, the cells were flattened and polygonal in shape, and most cells had S-100 protein immunoreactivity (b), while those cells immunostained with non-immune mouse serum did not show immunoreactivity (c). Magnifications are identical ($\times 150$) in (a), (b) and (c).

floating. The percentage of immunoreactive FS cells at this stage was estimated to be 7.6% (the mean of results for duplicate cultures) and the cells are shown in Fig. 1a. After 2 days in culture, some cells, especially those forming clumps, began to become flattened and polygonal in shape, this being evident in most cells after 6 days of culture. The cultures almost reached confluency after 7 days, and dome-like structures were observed (Fig. 2). The cultures were then passed and successively incubated. Cell viability after the passage procedure was about 90%. At this time, cells began to become flattened and polygonal in shape soon after they were attached to the surface. After the second passage on day 14, most cells had S-100 protein immunoreactivity (Fig. 1b), while cells stained with non-immune mouse serum lacked non-specific staining (Fig. 1c). These cultures were therefore estimated to be FS cell-enriched cultures, and FS cell-conditioned medium (FSCM) was collected.

Effects of FSCM on the action of activin A

Effects of FSCM on the number of FSH cells and on the action of activin A were examined by means of primary cultures of anterior pituitary cells. First, cells were cultured for 3 days in a medium containing several concentrations of FSCM in the absence of activin A. No significant differences were found between FSCM-treated and control cultures in the number of FSH cells (Fig. 3a), indicating that FSCM did not singly affect the number of FSH cells in the examined range of $0.1\text{--}100\text{ }\mu\text{l/ml}$. Second, cells were cultured for 3 days in the simultaneous presence of

Fig. 2. Light micrograph of cells after 7 days in culture. Regions of the monolayer contained dome-like structures (D) raised above the surface of the dish.

Fig. 3. Effects of folliculo-stellate cell-conditioned medium (FSCM) on the action of activin A. Dispersed anterior pituitary cells were pre-cultured for 1 day, and then cultured for 3 days in fresh medium containing FSCM alone (a), or activin A and FSCM (b). Immunostained cells were counted at the light microscopic level, and percentages of immunoreactive FSH cells are presented as the mean $\pm$ SEM of results for triplicate wells. Similar results were obtained in three (a) or seven (b) independent experiments. Ct, control; Act, activin A (10 ng/ml) alone; Act+FSCM, simultaneous presence of activin A (10 ng/ml) and several concentrations of FSCM. a, $P < 0.01$ vs. control; b, $P < 0.01$ vs. activin A alone.

activin A (10 ng/ml) and several concentrations of FSCM. FSCM significantly ($P < 0.01$) attenuated the stimulatory effect of activin A on the number of FSH cells in a concentration dependent manner. The inhibitory effect of FSCM reached its maximum at 10 μ l/ml, when the number of FSH

Fig. 4. Comparison of the effects of folliculo-stellate cell-conditioned medium (FSCM) and control conditioned medium (D1CM) on the action of activin A. After 1-day pre-culture, cells were cultured for 3 days in fresh medium containing activin A (10 ng/ml) and FSCM (10 μ l/ml), or activin A (10 ng/ml) and D1CM (10 μ l/ml). Percentages of immunoreactive FSH cells are presented as the mean $\pm$ SEM of results for triplicate wells. Similar results were obtained in two independent experiments. Ct, control; Act, activin A alone. a, $P < 0.01$ vs. control; b, $P < 0.01$ vs. activin A alone.

cells was reduced to the control level. The number of FSH cells did not decrease to beneath the control level even when the concentration of FSCM was increased to 100 μ l/ml (Fig. 3b). In contrast to FSCM, D1CM (10 μ l/ml) did not reduce the stimulatory effect of activin A (Fig. 4). This inhibitory effect on the action of activin A was also observed in the conditioned media from the other two FS cell-enriched cultures (data not shown).

Effects of the antibodies on the inhibitory action of FSCM on activin A

The effects of anti-follistatin, anti-S-100 protein and anti-bFGF on the suppressive effect of FSCM on the action of activin A were examined. The number of FSH cells, significantly ($P < 0.01$) higher in activin A-treated than in control cultures, was reduced to the control level by incubation with activin A plus FSCM, as already shown. Among the antibodies examined, anti-follistatin significantly ($P < 0.01$) reduced the inhibitory effect of FSCM on the action of activin A in a concentration dependent manner. 10 μ l/ml of anti-follistatin returned the number of FSH cells to the level in

Fig. 5. Effects of the antibodies to follistatin, bFGF and S-100 protein on the inhibitory effect of FSCM on the action of activin A. After 1-day pre-culture, the cells were cultured for 3 days in fresh medium containing activin A and FSCM, in the presence of anti-follistatin (0.1, 1 or 10 $\mu\text{l/ml}$) (a), or either anti-follistatin (anti-F, 10 $\mu\text{l/ml}$), anti-bFGF (10 $\mu\text{l/ml}$) or anti-S-100 protein (10 $\mu\text{l/ml}$) (b). Percentages of immunoreactive FSH cells are presented as the mean $\pm$ SEM of results for triplicate wells. Similar results were obtained in two independent experiments. Ct, control; Act, activin A (10 ng/ml) alone; Act+FSCM, activin A (10 ng/ml) and FSCM (10 $\mu\text{l/ml}$); Act+FSCM+, activin A (10 ng/ml), FSCM (10 $\mu\text{l/ml}$) and the indicated antibody. a, $P < 0.01$ vs. control; b, $P < 0.01$ vs. activin A alone; c, $P < 0.01$ vs. activin A plus FSCM.

cultures treated with activin A alone, thus absolutely neutralizing the effect of FSCM (Fig. 5a). By contrast, the other antibodies, anti-bFGF and anti-S-100 protein, did not significantly influence the effect of FSCM at a concentration of 10 $\mu\text{l/ml}$ (Fig. 5b).

Fig. 6. Effects of follistatin on the action of activin A. After 1-day preculture, cells were cultured for 3 days in fresh medium containing follistatin alone (a), or activin A and follistatin (b). Percentages of immunoreactive FSH cells are presented as the mean $\pm$ SEM of results for triplicate wells. Similar results were obtained in three independent experiments. Ct, control; Act, activin A (10 ng/ml) alone; Act+follistatin, simultaneous presence of activin A (10 ng/ml) and several concentrations of follistatin. a, $P < 0.01$ vs. control; b, $P < 0.05$ vs. activin A alone; c, $P < 0.01$ vs. activin A alone.

Effects of follistatin on the action of activin A

In order to confirm that follistatin was the active substance in FSCM which suppressed the expression of the action of activin A, the effects of follistatin on the number of FSH cells and on the action of activin A were examined. When cells were cultured for 3 days in the medium containing

follistatin in the absence of activin A, the number of FSH cells showed a tendency to decrease from the control level as the concentration of follistatin was increased (1.6–40 ng/ml), but no significant differences were found even if the concentration of follistatin was raised to 200 ng/ml (5.7 nM) (Fig. 6a). On the other hand, when cells were cultured for 3 days in the simultaneous presence of activin A (10 ng/ml) and follistatin (0.4–50 ng/ml), follistatin significantly ($P < 0.01$) inhibited the increase in the number of FSH cells caused by activin A. The inhibitory effect of follistatin was concentration dependent, and 50 ng/ml (1.4 nM) follistatin reduced the number of FSH cells to the control level (Fig. 6b). It was therefore shown that follistatin mimicked the inhibitory effect of FSCM on the action of activin A.

Discussion

In the present study, we found that the media conditioned from the FS cell-enriched cultures suppressed the stimulatory effect of activin A on the number of FSH cells [13, 14]. This confirms the paracrine effects of FS cells [35, 36] and indicates that FS cells can also affect the function of FSH-gonadotropes by inhibiting the action of activin A on these cells.

Pituitary FS cells produce several bioactive molecules, such as S-100 protein [21], bFGF [22], vascular endothelial growth factor [23, 24], follistatin [24] and interleukin-6 [25]. In the present study, anti-follistatin, but not anti-S-100 protein nor anti-bFGF, neutralized the inhibitory effect of the conditioned media on the action of activin A. Furthermore, the effect of the conditioned media was mimicked by follistatin. These observations indicate that the active substance from FS cells was follistatin. In addition, it is unlikely that some other molecules present in the conditioned media were required for this action of follistatin, for example as cooperators, because follistatin alone completely mimicked the inhibitory effect of the conditioned media. Taken together, our findings indicate that follistatin is the paracrine factor which is secreted from FS cells and suppresses the expression of the growth factor-like action of activin A on the FSH cell-lineage. This hypothesis is supported by the recent findings that follistatin binds to activin A [26] and forms an inactive

complex with neither stimulatory nor inhibitory effects on FSH secretion from cultured pituitary cells [37].

While the effects of the conditioned media from FS cells or follistatin on FSH secretion were not examined, neither of them was shown to manifest inhibitory effects on the number of FSH cells when added to the cultures in the absence of activin A. Since follistatin was originally isolated as an inhibitor of FSH secretion [38–40], these results suggest that the mechanisms involved in the regulation of FSH secretion and of the number of FSH cells may be different. This concept is consistent with our previous results which demonstrated the possibility that different modes of action of activin A may be involved in the regulation of FSH secretion and of the number of FSH-gonadotropes [14]. Corrigan *et al.* recently proposed that the inhibitory effects of follistatin and inhibin on FSH secretion might depend on their ability to interfere with the activity of endogenous activin [17]. It can therefore be assumed that endogenous activin in the pituitary, whose action can be inhibited by follistatin, is involved in basal FSH secretion but not in the differentiation or proliferation of the FSH cell-lineage.

The topographical distribution of FS cells in the pituitary *in situ* is known to change according to physiological and experimental conditions. For example, FS cells closely surround gonadotropes after gonadectomy but not thyrotropes after thyroidectomy in the anterior pituitary of rats [41]. It is classically known that hypertrophic “castration cells” which have the properties of gonadotropes appear following destruction of seminiferous tubules or castration [42]. As it is possible that activins from some sources [15] are responsible for the appearance of gonadotropic castration cells, the effect of FS cells demonstrated in this study might be involved in stopping castration cells from increasing any more to keep the pituitary function within the physiological extent. Studies on the anatomic distribution of activins and the activin-related proteins [15, 16, 43] and on the regulation of their gene expression [44–46] have given support to the physiological importance of these materials. In this sense, the results described above provide additional support for this by demonstrating a novel regulatory pathway of the action of activin A including the involvement of *in situ*

components of the pituitary gland.

In summary, the present study has demonstrated that the conditioned media from pituitary FS cells suppress the stimulatory effect of activin A on the number of FSH cells, and that this paracrine action is mediated by follistatin. These results suggest that FS cells can affect the function and/or the morphogenesis of the FSH cell-lineage of the pituitary gland in a paracrine way by affecting the action of activin A on these cells.

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