Abstract

Immunohistochemistry using an antiserum raised against the synthetic follistatin peptide (residues 123–134) was used, in the present study, to detect the stage-specific appearance of immunoreactive follistatin in the rat testis. Follistatin immunoreactivity was not found in Sertoli and Leydig cells, while it was clearly detected in spermatogenic cells. Follistatin-like immunoreactivity was detected in the cytoplasm and nucleus of late pachytene spermatocytes. Although the reaction in the cytoplasm disappeared after meiosis, it continued to be intense in the nucleus from pachytene spermatocytes to round spermatids. This finding indicated that follistatin or its closely related peptide produced in late pachytene spermatocytes migrates from the cytoplasm to the nucleus. We subjected rat testis homogenate to affinity chromatography on a sulfate-cellulofine and anti-follistatin Cys (123–134)–Affi-Gel Hz column followed by reverse-phase HPLC and analyzed the resulting fractions by Western blotting using follistatin antiserum. Three major bands at 57, 45 and 39 kDa or four bands at 52, 44, 39 and 34 kDa were detected in crude preparations from rat testis homogenate, under reducing or non-reducing SDS-PAGE respectively. The protein from rat testis, which was recognized by anti-follistatin (123–134) antiserum, exhibited a characteristic pattern for follistatin on SDS-PAGE, i.e. slower migration under reducing conditions than under non-reducing conditions, suggesting that it was follistatin or its closely related protein. Follistatin or its closely related protein may be a stage-specific modulator of spermatogenesis. Since follistatin-like immunoreactivity was not found in oocytes in any stage of development from embryonic to adult rats, it may act in an event specific to spermatogenesis, such as nuclear condensation.

European Journal of Endocrinology 137 523–529

Introduction

Activin, a member of the transforming growth factor (TGF)-β family, was initially isolated from gonads as a stimulator of follicle-stimulating hormone (FSH) secretion from the pituitary gland. It was found to be a dimer of the β subunit of inhibin which was an inhibitor of FSH secretion (1–4). Follistatin, originally obtained from porcine follicular fluid during the isolation of inhibin and activin, is structurally different from inhibin/activin in spite of its inhibin-like activity (5, 6). Activin and follistatin produced in the gonads were thought to serve as modulators of FSH release in a long loop feedback manner to the pituitary. However, their broad distribution throughout the body, including the pituitary gland (7, 8), suggests that they act in a paracrine mechanism.

Like other members of the TGF-β family, activin exhibits potent activities in diverse biological processes, including promotion of erythroid differentiation (9), induction of mesoderm tissue formation (10), stimulation of early embryogenesis (11), promotion of folliculogenesis (12), modulation of pituitary and pancreatic hormone release (13–15), and bone formation (16).

Nakamura et al. (17) found that follistatin is an activin-binding protein. Follistatin binds to both inhibin and activin through the common β subunit (18). Neutralization of activin’s activity by follistatin is proven in various systems, such as the stimulation of FSH secretion in cultured pituitary cells (8), induction of mesoderm tissue formation in Xenopus oocytes (19), and differentiation of rat granulosa cells (20). These results indicated that follistatin is a functional regulator of activin’s activity.

Several investigations have been carried out to clarify the role of inhibin and activin in spermatogenesis (21–23). Inhibin reduces spermatogonial number (21), and activin stimulates spermatogonial proliferation in vitro (22). While the studies have shown the significance of inhibin/activin, the function of follistatin in spermatogenesis is still unclear.

To detect the localization of follistatin in the rat testis, the present study was undertaken by immunohistochemistry.
using an antiserum raised against the synthetic peptide of follistatin.

Materials and methods

Materials

Recombinant human follistatin (rhFS) was kindly provided by Dr M Muramatsu (Department of Biochemistry, Faculty of Medicine, Saitama Medical School, Japan) and Dr Y Eto (Ajinomoto Co. Inc., Central Research Laboratories, Kawasaki, Japan). Avidin–biotin–peroxidase (ABC) reagent was obtained from Vector Laboratories Inc. (Burlingame, CA, USA). The peptide fragment of residues 123–134 of human follistatin and diphtheria toxoid-conjugated Cys (123–134) of human follistatin were obtained from Chiron Memetopes Peptide Systems (Victoria, Australia). CNBr-activated Sepharose 4B was obtained from Pharmacia LKB (Uppsala, Sweden). The Affi-Gel Hz and Econo-Pac 10DG desalting column were from BioRad (Richmond, CA, USA). Sulfate-cellulofine was from Seikagaku Kogyo (Tokyo, Japan). The HPLC STMS-300 column (Cosmosil Packed Column, 4.6×250 mm) was obtained from Nacalai Teque (Kyoto, Japan). ECL Western blotting detection reagents were obtained from Amersham International plc (Amersham, Bucks, UK).

Animals

Wistar Imamichi strain rats were obtained from the Imamichi Institute for Animal Reproduction (Ibaraki, Japan). All animals were maintained in air-conditioned quarters and food and water were available ad libitum.

Preparation of tissue sections

For immunohistochemical examinations, the testes were excised from 4-, 6-, 8-, 12- and 18-day plus 6-, 8- and 10-week-old rats (four animals were used at each age). Under ether anesthesia, the testes were excised from 4-, 6-, 8- and 12-day-old rats and were fixed in Bouin’s fixative for 4 to 6 h. Older groups (18-day, 6-, 8- and 10-week-old rats) were similarly anesthetized and perfused transcardially with saline followed by Bouin’s fixative. Testes excised from each animal were sliced into slabs and immersed in the same fixative for 4 to 6 h. After fixation, tissues were dehydrated in a graded ethanol series, cleared in xylene and embedded in a paraffin block. Sections were cut on a sliding microtome and then subjected to immunohistochemical procedure using follistatin antiserum.

Immunohistochemical procedure

A rabbit polyclonal antiserum against synthetic follistatin peptide (residues 123–134) (24–26) was used for immunohistochemistry. The amino acid sequence of residues 123–134 is identical in human (6), rat (27), mouse (28), ovine (29), bovine (30) and porcine (31) follistatin. The antiserum was purified by affinity chromatography on protein A–Sepharose before performing immunohistochemistry.

The ABC procedure was performed as described previously (32). After deparaffinization, the sections were incubated in H2O2 in methanol for 30 min to eliminate endogenous peroxidase. They were placed in a moist chamber, covered first with 5% normal goat serum for 30 min to minimize non-specific staining, followed by rabbit antiserum diluted 1:1200 with 0.3% BSA/0.01 mol/l PBS for overnight incubation at room temperature. After washing with 0.01 mol/l PBS, they were incubated with biotinylated goat anti-rabbit IgG for 30 min and reacted with ABC reagent for 45 min. A mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 0.01 mol/l PBS–0.01% H2O2 was added for 7 to 10 min to develop the peroxidase reaction.

To confirm the method and antiserum specificity, control sections were incubated with non-immune rabbit serum from the same rabbit from which the antiserum was obtained or primary antiserum absorbed with excess (400 ng) rhFS. Additional controls were stained without secondary antiserum or ABC reagent.

Preparation of affinity chromatography column

The peptide fragment residues Cys (123–134) of human follistatin coupled with diphtheria toxoid was given to adult rabbit as an antigen. The rabbit was injected with 500 mg of the conjugate emulsified in Freund’s complete adjuvant every 2 weeks. Serum was collected by heart puncture 5 weeks after first immunization. The serum was purified by affinity chromatography on follistatin peptide (123–134)-coupled Sepharose 4B column. The antiserum recognized both follistatin from porcine follicular fluid (pFS) and rhFS in SDS-PAGE and immunoblotting analysis (data not shown). IgG from serum was partially purified by ammonium sulfate precipitation, and then coupled to Affi-Gel Hz.

Preparation of testicular extract

The testes excised from 20 adult rats (8 to 10 weeks old) were homogenized in buffer I (20 mmol/l Tris–HCl buffer, pH 7.3, containing 2 mmol/l EDTA, and 5 mmol/l benzamidine–HCl) containing 0.15 mol/l NaCl, 1 mmol/l di-isopropylfluorophosphate, 1 mmol/l N-ethylmaleimide, and 1 mmol/l phenylmethylsulphonyl fluoride. The homogenates were centrifuged at 1600 g for 15 min. NaCl and Triton X-100 were added to the supernatant to make the final concentrations of 0.35 mol/l and 1% respectively. The resulting solution was stirred for 30 min and centrifuged at 9900 g for 30 min. Sulfate-cellulofine (10 ml) was added to 20 ml...
of each supernatant and stirred gently for 12 h. The gels were washed with buffer I containing 0.35 mol/l NaCl and 0.1% Triton X-100, resuspended in the same buffer, and packed into the column (1.6 × 20 cm). The column was then eluted with buffer I containing 1.5 mol/l NaCl and 0.1% Triton X-100. The elute was dialyzed against 10 mmol/l Tris, pH 7.5, overnight, and then applied to the anti-follistatin–Affi-Gel Hx column. The column was

Table 1 The immunoreaction of testicular cells with anti-follistatin (123–134) at various ages.

<table>
<thead>
<tr>
<th>Cells</th>
<th>4 days</th>
<th>6 days</th>
<th>8 days</th>
<th>12 days</th>
<th>18 days</th>
<th>6 weeks</th>
<th>8 weeks</th>
<th>10 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatogenic cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatogonia</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spermatocyte</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Round spermatid</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Elongated spermatid</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Somatic cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sertoli cells</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Leydig cells</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The intensity of reaction is given on a scale of – to +++: (−) negative, (+) moderately positive, (+++) intensely positive. The symbol / means the absence of corresponding cells.

Figure 1 Histochemical micrographs showing localization of follistatin-like immunoreactivity in the rat testis (bar = 50 μm). (A) Moderate immunoreaction with anti-follistatin (123–134) serum was found in the cytoplasm and nucleus of the spermatocyte and an intense reaction was found in the nucleus of the round spermatids, whereas it was not detected in the spermatogonia in the 6-week-old rat. (B) Although follistatin-like immunoreactivity was not detected in pachytene spermatocytes, it was intense in the nucleus but not in the cytoplasm of round spermatids in stages I to VI of seminiferous epithelia in the 8-week-old rat. (C) In stages VII to VIII, follistatin-like immunoreaction was found in both the cytoplasm and nucleus of pachytene spermatocytes. Intense immunoreaction was also found in the nucleus of round spermatids in stage VI to VII (8-week-old rat). (D) In stages XI to XII, the reaction was found to be intense in the nucleus and moderate in the cytoplasm of pachytene spermatocytes (8-week-old rat). When the antiserum was absorbed with an excess amount (400 ng) of rhFS, the reaction was not found in any type of cell in the testis.
washed with 10 mmol/l Tris–HCl, pH 7.5, followed by 10 mmol/l Tris–HCl, pH 7.5/0.5 mol/l NaCl, and then eluted by 100 mmol/l glycine, pH 2.5. The eluate was desalted by using an Econo-Pac 10DG column (4.6 × 250 mm), equilibrated with 0.1% trifluoroacetic acid (TFA). The desalting sample was injected into a 5TMS-300 column (4.6 × 250 mm), equilibrated with 0.1% TFA, and eluted with a linear gradient of 16–40% (v/v) acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min. The eluted fractions were examined by SDS-PAGE and immunoblotting analysis using anti-follistatin (123–134).

SDS-PAGE and immunoblotting

pFS was purified from follicular fluid as described previously (8). The pFS, rhFS and aliquots of eluted fractions were dissolved in SDS-PAGE sample buffer (10 mmol/l Tris, 1% SDS, 2% β-mercaptoethanol and 0.04% bromophenol blue) and heated at 100°C for 5 min. Electrophoresis was carried out in 12.5% gels as described by Laemmli (33), and the protein was transferred to Immobilon membranes (Millipore). The membrane was blocked with Block Ace (Dainihonsei-yaku Inc., Osaka, Japan) and incubated with anti-follistatin (123–134) serum diluted 1:3000 with 0.3% BSA/0.3% Tween-20/0.01 mol/l PBS overnight at room temperature. After washing in 0.01 mol/l PBS/0.3% Tween-20, the membranes were incubated for 2 h with horse-radish peroxidase-labeled goat anti-rabbit IgG (Vector Laboratories Inc, Burlingame, CA, USA). Immunoreactive protein bands were detected using the enhanced chemiluminescence detection method (ECL Western blotting detection reagent; Amersham International plc). To determine non-specific blots, the antiserum prepared with excess rhFS (1–2 μg) was used in a parallel experiment.

Results

Localization of follistatin-like immunoreactivity in rat spermatogenic cells

The immunoreactions of testicular cells with anti-follistatin (123–134) are summarized in Table 1. In this study, follistatin-like immunoreactivity was not detected in the testis from 4- to 18-day-old rats. In 6-week-old rats, although matured spermatids were not observed, spermatocytes and round spermatids were found in some testicular tubules. A moderate immunoreaction with anti-follistatin (123–134) was found in the cytoplasm and the nucleus of spermatocytes and in the nucleus of round spermatids, whereas it was not detected in spermatogonia (Fig. 1A). Follistatin immunoreactivity was not detected in Sertoli and Leydig cells in 6-week-old rats.

In mature rats (8 and 10 weeks old), the reaction with anti-follistatin (123–134) serum was detected in both nucleus and cytoplasm of late pachytene spermatocytes (Fig. 1C and D). The immunoreactivity was also detected in the nucleus of round spermatids but was not in their cytoplasm (Fig. 1B, C and D). When the antiserum was absorbed with an excess amount (400 ng) of rhFS, the reaction was abolished. Immunoreactivity of follistatin was not detected in either Sertoli or Leydig cells.

Figure 2 summarizes our results obtained from histochemical analyses with anti-follistatin (123–134) serum in testicular sections from mature rats. Spermatogonia were not stained at any stage examined in this
study. No reaction was found in preleptotene to zygote spermatocytes. Pachytene spermatocytes were not stained in stages I to V but did stain in stages VI to XII. The reaction in spermatocyte cytoplasm disappeared in the diplotene phase (stage XIII). Immunoreactivity in the pachytene spermatocyte nucleus was slight in stage VI, moderate in stages VII to IX, and intense in stages X to XII. Although the reaction in the cytoplasm disappeared after meiosis, it continued to be intense in the nucleus from pachytene spermatocytes to round spermatids. The reaction in nuclei appeared to be weaker in step 8 spermatids and disappeared in step 10. The reaction was not found in spermatids after step 10 or in spermatozoa.

**Western blotting analysis of follistatin-like immunoreactivity in the rat testis**

We subjected rat testis homogenate to affinity chromatography on sulfate-cellulofine and anti-follistatin Cys (123–134) column followed by reverse-phase HPLC and analyzed the resulting fraction by Western blotting using anti-follistatin (123–134) serum. Follistatin immunoreactivity with the crude preparation from the homogenate was detected in fraction 11 to 14 (data not shown).

The results obtained in Western blotting analysis are shown in Fig. 3. When the anti-follistatin (123–134) was used for immunoblotting under reducing conditions, pFS and rhFS exhibited three major bands at 41, 39 and 37 kDa (lane 1), and at 42, 40 and 39 kDa (lane 2) respectively. In the preparation from rat testis homogenate, three major bands at 57, 45 and 39 kDa were detected with anti-follistatin (123–134) (lane 3). Under non-reducing conditions, four bands were detected at 35, 32, 30 and 28 kDa for pFS (lane 4) and at 37, 35, 34 and 32 kDa for rhFS (lane 5). Four bands at 52, 44, 39 and 34 kDa were detected in the preparation from rat testis homogenate (lane 6). When the antiserum was absorbed by the excess of rhFS (1–2 µg), the immunoreaction was markedly reduced.

**Discussion**

The present study demonstrated that follistatin-like immunoreactivity is located in rat spermatogenic cells by immunohistochemistry. During the development of spermatogenic cells, follistatin-like immunoreactivity is first detected in the cytoplasm and nucleus of late pachytene spermatocytes. Although the reaction in the cytoplasm disappeared after meiosis, it continued to be in the nucleus from pachytene spermatocytes to round spermatids. This finding suggests that follistatin or its closely related protein produced in late pachytene spermatocytes migrates from the cytoplasm to the nucleus. A previous study has demonstrated that the level of follistatin mRNA signal is high at stages IX to XI of the cycle (34). This is in agreement with our results which show a high level of follistatin-like immunoreactivity in cytoplasm of spermatocytes at stages X to XII. However, Kaipia et al. (34) showed follistatin mRNA expression in Sertoli cells. The possibility still remains that follistatin is produced in Sertoli cells and then rapidly taken up by spermatocytes. In previous studies, the immunoreactive βA subunit of inhibin/activin has been detected in the cytoplasm (35) and the nucleus of spermatogenic cells (36). It is, therefore, conceivable that follistatin binds to activin in the cytoplasm and nucleus of spermatogenic cells.

Follistatin-like immunoreactivity was detected in spermatogenic cells from meiotic prophase to the period just before initiation of nuclear condensation. Because inhibin and activin act in oocyte meiotic resumption (37, 38), follistatin may also act in meiotic regulation during spermatogenesis. In females, however, follistatin-like immunoreactivity was not found in oocytes in any stage of development from embryonic to adult rats (K Ogawa, unpublished observations), whereas it was detected in granulosa cells in preovulatory follicle by the same antiserum (25). It is, therefore, conceivable that follistatin may act in an event specific to spermatogenesis, such as nuclear condensation.

The results of Western blot analyses indicated the existence of follistatin or its closely related protein in rat testis. A protein extracted from rat testis, which was recognized by anti-follistatin (123–134) antiserum, exhibited a characteristic pattern for follistatin on SDS-PAGE, i.e. slower migration under reducing conditions than under non-reducing conditions (17).
In addition to activin-binding activity, follistatin has been reported to have affinity for nucleic acid-like substances, such as heparin (7), dextran sulfate (8) and heparan sulfate (39). Taken together with the finding that follistatin-like immunoreactivity is located in the nucleus, follistatin may be a DNA-binding protein of spermatogenic cells. Postmeiotic male germ cell differentiation to mature spermatozoon involves remarkable structural and biochemical changes, including nuclear DNA compaction and acrosome formation. Expression of various genes, such as protamine (40), caliphermin (41) and transition protein-1 (40, 42), is thought to be required for this process. In this study, follistatin-like immunoreactivity was highly accumulated in the nucleus in postmeiotic germ cells, suggesting that it may be associated with a transcriptional activator, such as the cyclic AMP-responsive element modulator (40–42), particularly during the haploid phase. It may be responsible for the activation of several haploid cell-specific genes. Further observations will be needed to clarify this point.

In conclusion, follistatin-like immunoreactivity was detected in both the nucleus and cytoplasm in spermatogenic cells, and the immunoreactiviy varied in intensity depending on the stage of the cycle. Follistatin or its closely related protein may be a stage-specific modulator of spermatogenesis.

Acknowledgements
The authors wish to thank Dr M Muramatsu and Dr Y Eto for providing rhFS.

References


Received 17 February 1997
Accepted 7 April 1997