0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 9
Printed in U.S.A.

Hormonal Dependency of Neural Cadherin in the Binding of Round Spermatids to Sertoli Cells *in Vitro**

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ABSTRACT

The procession of round spermatids through stages VII and VIII of the rat spermatogenic cycle is critically dependent on testosterone (T). When intratesticular T levels are reduced, round spermatids appear to slough from the seminiferous epithelium, resulting in the disappearance of elongated spermatids. We hypothesized that T-dependent cell adhesion molecules are involved in Sertoli cell-round spermatid interactions. This study examined the hormonal regulation of one candidate cell adhesion molecule, N-cadherin, *in vitro* and its participation in Sertoli cell-round spermatid adhesion in coculture. Sertoli cells were isolated from 20-day-old Sprague-Dawley rats; treated with FSH and T, alone or in combination; and incubated for 48 h before determination of N-cadherin concentrations in Sertoli cell extracts by RIA. Both FSH and T significantly increased the cellular content of N-cadherin (3.7-fold), whereas FSH or T alone had no effect. Round spermatids were isolated from adult rats, and their adhesion to Sertoli cells was assessed in a 48-h coculture in the presence of FSH, T, or FSH plus T. Adherent round spermatids were quantitated by histological evaluation after staining with the periodic acid-Schiff reaction. A dose-dependent increase in round spermatid density (number of round spermatids bound per 10,000-μm^2 Sertoli cell culture surface area) was observed with increasing T doses (7–28 ng/ml) in the presence of FSH (1 μg/ml), whereas FSH and T alone at these doses produced no effect. T also increased the N-cadherin content of the cocultures in a dose-dependent manner in the presence of FSH. Addition of an N-cadherin antiserum to the Sertoli cell-round spermatid coculture in the presence of FSH and T significantly ($P < 0.0001$) reduced round spermatid density by 65%. It is concluded that both the production of N-cadherin by Sertoli cells and the binding of round spermatids to Sertoli cells are stimulated in a synergistic manner by T and FSH. Furthermore, the immunoneutralization data suggest the active involvement of N-cadherin in round spermatid-Sertoli cell adhesion *in vitro*. N-Cadherin may be one of the factors that subserve the androgen-dependent process of round to elongated spermatid maturation. (*Endocrinology* **137:** 3877–3883, 1996)

THE RELATIVE roles of testosterone (T) and FSH in spermatogenesis remain controversial. The experimental lowering of intratesticular T levels leads to the disappearance of elongated spermatids in the testis (1–6). We have shown that the principal spermatogenic lesion in this setting is the loss of round spermatids from the seminiferous epithelium, and more precisely, that the maturation of round spermatids between stages VII and VIII is highly T dependent (6). Premature sloughing of round spermatids from the seminiferous epithelium was suggested by the observation that within 3 weeks of T suppression treatment, round spermatids were seen in the lumen of the epididymis (7). It has been proposed that T may exert its action on spermiogenesis by the induction of cell adhesion molecules (CAM) present at the intercellular junction between Sertoli cells and round spermatids (5, 7).

A variety of intercellular junctions exist between Sertoli cells and germ cells (for review, see Ref. 8). In the case of spermatids, the type of junction is dependent upon the stage of maturation, with desmosomal gap junctions being involved in binding of spermatids before step 8, whereas the unique Sertoli cell ectoplasmic specialization (ES) junction is formed between Sertoli cells and spermatids from step 8 onward. These junctions have been well characterized structurally *in vivo* (8–10) and *in vitro* (11). In contrast, the involvement and regulation of CAMs potentially involved in these junctions are poorly described, although a number of CAMs have been localized to the testis (12–17).

One such CAM is neural (N-) cadherin, a transmembrane glycoprotein that mediates calcium-dependent, homotypic, intercellular adhesion (for review, see Ref. 18). Cadherin was immunolocalized in the seminiferous epithelium to patches over Sertoli cell and spermatogenic cell membranes and at certain stages, corresponding to areas of ES in Sertoli cells (15, 19). N-Cadherin messenger RNA (mRNA) was detected in the testis (13, 16, 20) and was shown to be hormonally regulated by estrogen in the immature mouse (21).

The structural interaction between round spermatids and Sertoli cells was studied *in vitro* (11, 22), and mature ES was shown to develop during coculture. Spermatid adhesion showed a dose-dependent increase with T only when FSH was present. The mechanisms of FSH and T actions may differ, as, for example, it was proposed that FSH induced the peripheral distribution of cytoskeletal proteins involved in binding (11, 22). However, the mechanism of action of T on adhesion remains unclear.

N-Cadherin is a good candidate for involvement in round spermatid-Sertoli cell adhesion, yet to date, there have been no reports of its regulation by FSH or studies correlating the induction of N-cadherin expression with spermatid-Sertoli

Received March 12, 1996.
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* This work was supported by the National Health and Medical Research Council of Australia (Grant 943208).

cell adhesion *in vitro*. The current study examines the effects of FSH and T on round spermatid adhesion *in vitro*, in particular their effects on N-cadherin levels in Sertoli cells. The data suggest that levels of N-cadherin in the Sertoli cell are hormonally regulated by both FSH and T, and immunoneutralization of N-cadherin inhibits the binding of round spermatids to cultured Sertoli cells.

Materials and Methods

Hormones

Highly purified hFSH (HPAC 84/1; 2350 IU/mg by FSH RRA) was obtained from the Human Pituitary Advisory Committee (Canberra, Australia). T was obtained from Sigma Chemical Co. (St. Louis, MO) and diluted as required in DMEM from a stock solution of 1 μg/ml in ethanol.

Sertoli cell isolation

Sertoli cells were isolated from 20-day-old male Sprague-Dawley rats using the method of Dorrington *et al.* (23). After isolation, Sertoli cells were suspended in DMEM (supplemented with 1 mM L-glutamine, nonessential amino acids, and sodium bicarbonate) and 10% FCS. Sertoli cells were then plated into 24-well tissue culture plates at a concentration of 2.5×10^6 cells/well. Culture plates were precoated 4 h before use with Matrigel (Collaborative Research, Waltham, MA; lot 903015; 14.4 mg/ml) at a 1:8 dilution in DMEM (40 μl/well). After a 48-h incubation at 37 C, Sertoli cells underwent a hypotonic shock treatment with 10% DMEM in water for 90 sec to remove any contaminating germ cells (24). Sertoli cells were then cultured for an additional 24 h with DMEM-0.1% BSA before incubation with hormones for up to 4 days. Sertoli cells were solubilized for 2 h with 0.5% (wt/vol) Na deoxycholate (DOC) in Dulbecco's phosphate buffer [10 mM $NaH_2PO_4 \cdot H_2O$-Na_2HPO_4, 154 mM NaCl (pH 7.4), and 0.68 mM $CaCl_2$; 250 μl/well] containing protease inhibitors (one tablet per 50 ml buffer; Protease Inhibitor Cocktail, Boehringer Mannheim, Mannheim, Germany). The use of DOC solubilized the entire Sertoli cell culture and has been shown to quantitatively solubilize N-cadherin in whole tissue extracts (25). The solubilized Sertoli cell extract was then stored at 4 C before assay in the N-cadherin or PAN-cadherin RIAs. To ensure that an even number of Sertoli cells was present in each well, cell numbers from four control wells per plate were assessed by a neutral red cell quantitation assay at the end of each culture (26). In a typical culture, $1.25–5 \times 10^6$ Sertoli cells/well gave an OD range at 450 nm of 0.25–0.48, with a within-well variation of 10%. To assess whether the Sertoli cell protein content varied with treatment, the washed cells obtained from cell cultures stimulated with graded doses of FSH (0–1 μg/ml) and a fixed dose of T (28 ng/ml) were solubilized in 0.1 M NaOH, and protein content was determined by the Bradford assay using BSA as standard. Cell protein content ranged between 14–15 pg/cell, with no significant differences between hormone doses. Cell viability (>85%) was determined by trypan blue dye exclusion. Sertoli cell purity was assessed at greater than 95% by immunocytochemistry at the end of the culture with polyclonal rabbit α-inhibin antiserum, which is specific for Sertoli cells, and a monoclonal mouse α-smooth muscle actin (Sigma), which will discriminate peritubular cells (27).

Isolation of round spermatids

Round spermatids (steps 1–9) were isolated from adult Sprague-Dawley rats (>60 days old). Briefly, two rats were killed by CO_2 inhalation, and testes were removed, decapsulated, and minced with a razor blade before dispersion by collagenase-trypsin treatment (28). After centrifugation, the cells were purified by elutriation, with round spermatids present in the 3′-fraction as defined by Loveland *et al.* (29). This fraction was then further purified on a Percoll (Pharmacia, Uppsala, Sweden) density gradient (30). Cells were washed and resuspended in McCoy's 5A medium supplemented with lactate, pyruvate, and sodium bicarbonate. The cell yield was $2.5–3 \times 10^6$ cells/four testes, with a viability of greater than 98%, as determined by trypan blue dye exclusion. Cells isolated by this procedure are routinely more than 90% pure, with minor contamination by pachytene spermatocytes and elongated spermatids, as assessed histologically (29).

Sertoli cell-round spermatid cell adhesion assay

Sertoli cells (4.5×10^5) were plated into 16-well chamber slides (Nunc, Naperville, IL), precoated with Matrigel (1:8 dilution in DMEM; 20 μl/well) 4 h before use. Incubation conditions and hypotonic shock are described above. According to the experimental protocol, purified round spermatids (60,000/well), hormonal treatment, and antiserum were added, and the coculture was incubated at 32 C for 48 h. At the completion of the incubation period, culture wells were washed five times with Dulbecco's phosphate buffer by hand pipetting and fixed in acetone for 30 sec. Binding of round spermatids to Sertoli cells was then quantitated as detailed below. The viability of the nonbound round spermatids was assessed after the 48-h incubation period and was routinely found to be 75–85%.

In immunoneutralization experiments, cocultures were incubated for 90 min with antiserum before washing five times and subsequent incubation with antiserum for 48 h. Treatment consisted of cocultures incubated with normal rabbit serum (NRS; 1:100), N-cadherin antiserum 6 (1:100), N-cadherin antiserum 6 preabsorbed with its immunizing peptide, and PAN-cadherin antiserum 7.

Cadherin antiserum production

Two polyclonal peptide antisera directed toward the cadherin molecule were raised in New Zealand White rabbits. The first antiserum was specific for the extracellular homotypic binding region of murine N-cadherin, peptide sequence FHLRAHAVDINGNQV [N-cadherin-(238–252)], as previously described (16, 31), but with an N-terminal tyrosine added to this sequence for iodination purposes. The second antiserum was directed toward the conserved cytoplasmic sequence common to all cadherins, peptide sequence DPTAPPYDSLLVFDYEG [N-cadherin-(852–868)] (31). Peptides were synthesized as C-terminal amides with an extra cysteine residue at the N-terminus and conjugated to diphtheria toxoid by the method of Lee *et al.* (32), using *N*-succinimidyl 6-maleimidocaproate (Fluka, Buchs, Switzerland) as the cross-linking agent. Peptide-carrier conjugates were emulsified in Freunds complete adjuvant and injected sc (three rabbits per immunogen). Subsequent boosts were undertaken with Freunds incomplete adjuvant. Rabbits were bled (~20 ml) from the marginal ear vein. Antisera bound iodinated N-cadherin or PAN-cadherin peptides (see below) with titers at 1:4000 from all rabbits. The N-cadherin antiserum 6 and PAN-cadherin antiserum 7 were used for all subsequent studies.

The specificities of the antisera, N-cadherin 6 and PAN-cadherin 7, were assessed by Western blotting. Normal rat testes were homogenized in 25 mM Tris-HCl, 0.32 M sucrose, and 2 mM $CaCl_2$, pH 7.2 with protease inhibitors (0.1 μM pepstatin, 2 mM *N*-ethyl maleimide, 1 μM leupeptin, and 0.1 mM phenylmethylsulfonylfluoride). The homogenate was spun initially at $800 \times g$ for 10 min at 4 C, and the supernatant was centrifuged at $12,000 \times g$ for 25 min. The pellet was extracted with 0.5% DOC on a rotating wheel for 70 min at 4 C and centrifuged at $35,000 \times g$ to remove particulate matter. The extract was electrophoresed on 10% polyacrylamide gels in SDS under nonreducing conditions and electrotransferred to nitrocellulose membranes. The nitrocellulose was incubated with the cadherin antisera at 1:100, and the bound antibody was detected with biotinylated antirabbit IgG (Silenus, Melbourne, Australia) followed by a streptavidin-horseradish peroxidase conjugate (Sigma). NRS was used as a control.

N-Cadherin RIA

The N-cadherin immunoactivity of Sertoli cells was assessed using a double antibody RIA procedure. The assay buffer used throughout was 10 mM phosphate buffer, pH 7.4, containing 0.154 M NaCl, 0.1% NaN_3, and 0.5% BSA. N-Cadherin peptide was used as standard (0.49–62.5 ng/ml) and iodinated by the chloramine-T procedure (33) for use as tracer. Iodinated peptide was immediately purified by reverse phase chromatography on a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) preequilibrated in 0.1% (vol/vol) trifluoroacetic acid (TFA), and the iodinated peptide was eluted with 0.1% (vol/vol) TFA-30% acetonitrile and stored at 4 C before assay. The N-cadherin antiserum (no. 6) was

used at a working dilution of 1:4000 in the presence of NRS (1:800). The incubation mixture, in a total volume of 400 μl (100 μl sample plus 100 μl assay buffer or 100 μl standard plus 100 μl 0.5% DOC in assay buffer, 100 μl tracer (10,000 cpm), and 100 μl N-cadherin antibody/NRS) was incubated overnight at room temperature. The precipitating second antibody was a goat antirabbit IgG diluted 1:80 in assay buffer, and the final separation of bound and free antibody used 6% polyethylene glycol in assay buffer (both incubated at 4 C for 30 min). Tubes were then centrifuged at 2500 × *g* for 45 min at 4 C, and the pellets were counted in a γ-counter. The interassay variation was 4.0% (n = 5), and the intraassay variation was 8.7% (n = 5). The slope of the standard curve after logit log-dose transformation was −1.11. The sensitivity was 0.058 ng N-cadherin peptide/tube, with an ED_{50} of 0.4 ng N-cadherin peptide/tube.

In addition to DOC, both Triton X-100 and SDS were investigated for their efficiency of N-cadherin solubilization, as assessed by RIA. Treatment of Sertoli cells with DOC resulted in maximal extraction of N-cadherin, whereas Triton was 3-fold less efficient, and N-cadherin was undetectable in SDS extracts. As the presence of DOC in the RIA caused an increase in tracer binding, all standard and samples tubes were standardized to a final DOC concentration of 0.125% (wt/vol). Samples solubilized in DOC were stable at 4 C for up to 18 days.

PAN-cadherin RIA

PAN-cadherin peptide was used as a standard (0.49–62.5 ng/ml) and iodinated by the lactoperoxidase procedure (34) for use as tracer. Iodinated peptide was purified in a similar manner as described for the N-cadherin peptide. The primary antibody was PAN-cadherin antiserum (no. 7) at a dilution of 1:4000. The incubation conditions and method were the same as those for the N-cadherin RIA. The interassay variation was 5.0% (n = 5), and the intraassay variation was 9.4% (n = 5). The slope of the standard curve after logit log-dose transformation was −1.26. The sensitivity of the assay was 0.078 ng PAN-cadherin peptide/tube, with an ED_{50} value of 0.3 ng PAN-cadherin peptide/tube.

Quantitation of round spermatid binding

Histochemical staining of Sertoli cell-round spermatid cocultures. Sertoli cell-round spermatid cocultures were histochemically stained using the periodic acid-Schiff reaction (35). Slides were counterstained in neat Harris' hemotoxylin before dehydration in a series of alcohol (70–100%) steps and were mounted using DPX (BDH, Poole, UK).

Counting method and binding analysis. Counting of round spermatids was performed on an Olympus BH-2 microscope (Olympus Corp., Melville, NY), using a ×100 oil objective. The image was captured using a F15 Panasonic video camera coupled to an Amiga 2000 computer using an Impact Vision 24 professional video adaptor (Great Valley Products, King of Prussia, PA). A software package (Grid 1.2, Graffitidata, Silkeborg, Denmark) was used to generate a 16-point coherent grid directly onto the video screen. The area associated with each point was 245 μm^2 at a final screen magnification of ×2727.

Fields to be counted were selected using a systematic uniform random sampling scheme (36) generated by a motorized stage (Lang and Co., Huttenberg, Germany). Briefly, fields were selected by stepping 300 μm to the right (X direction). The stage was then stepped in the Y direction, and a parallel set of fields was sampled. This scheme was continued until a total of 100 frames was sampled. If 80 round spermatids had not been counted in this sample, an additional 100 frames were counted to minimize counting error. Fields close to the edge of the well were not included in the analysis.

Round spermatids were counted when they were at least partially inside the counting grid but did not intersect the forbidden boundaries. Fields that included clumps of cells or did not have all 16 grid points covering Sertoli cells were excluded (~5–10% of the randomly selected fields) and occurred equally in all treatment groups. The number of spermatids bound to Sertoli cells was expressed as the number of round spermatids per 10,000 μm^2 Sertoli cell culture surface area (11, 22) and are henceforth termed round spermatid density.

Round spermatids were identified on the basis of their shape and size with a round to ovoid shaped nucleus. Only nondegenerating round spermatids ranging from 7.5–10 μm in diameter were counted. Pachytene spermatocytes, representing less than 1% of the cells bound to Sertoli cells, were identified by their large size and dispersed nuclear chromatin.

Gel permeation chromatography

Solubilized whole testis membranes were chromatographed on a Superdex HR200 gel permeation column (30 × 1 cm; Pharmacia, Piscataway, NJ) preequilibrated in 25 mM Tris, pH 7.2, containing 2 mM $CaCl_2$, 150 mM NaCl, and 0.1% (wt/vol) DOC at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected before determination of N-cadherin content by RIA. Solubilized Sertoli cells were not used for this experiment because the immunoactivity observed in the resultant fractions was below the sensitivity limit of the RIAs.

Statistical analyses

Parallelism between standard and unknown preparations in the N-cadherin and PAN-cadherin RIAs was established by assessing the slope values of a linearized dose response after logit log-dose transformation using standard bioassay statistical designs (37). Immunoassay data were analyzed using a four-parameter fit of the standard curve (Multicalc program, Wallac, Turku, Finland). Differences between groups were established using Peritz's F test for multiple comparisons among means (38). Significant differences were determined at the $P < 0.05$ level. All data are expressed as the mean ± 1 SD.

Results

RIA of N-cadherin

A RIA for N-cadherin was developed using an antiserum raised against the extracellular putative binding domain of rat N-cadherin [N-cadherin-(238–252)], iodinated N-cadherin as tracer, and N-cadherin peptide as standard. Serial dilutions of detergent-solubilized Sertoli cell extract gave parallel response curves with the standard (data not shown). Specificity was assessed from the apparent mol wt of intact N-cadherin protein after gel permeation chromatography of a solubilized whole testis extract (Fig. 1A). Two peaks of N-cadherin immunoactivity were observed, with a major peak of 138K consistent with intact rat N-cadherin (15, 16, 25, 31) and a minor peak of 250K. The 138K peak was also observed using the PAN-cadherin antiserum (Fig. 1B). The N-cadherin and PAN-cadherin antisera were examined by Western analysis of a rat testis extract. Three mol wt bands were detected at 138K, 79K, and 68K (data not shown). NRS controls showed a single band at 79K.

Production of N-cadherin by Sertoli cells in culture

To investigate the relationship between levels of N-cadherin produced by Sertoli cells and time in culture, Sertoli cells were incubated with either medium alone or a combination of FSH (1 μg/ml) and T (28 ng/ml) for 1, 2, 3, and 4 days before cell solubilization and RIA for N-cadherin (Fig. 2). In the absence of FSH and T, N-cadherin levels increased marginally after 2 days. In the presence of FSH and T, N-cadherin increased markedly to 350 pg/10^6 Sertoli cells by 4 days, with a maximal 3.7-fold increase (compared to control) by 2 days. Similar results were obtained with these hormones when Sertoli cells were cultured on plastic or Matrigel.

Cadherin concentrations were also determined in the same Sertoli cell extracts by the PAN-cadherin RIA. A similar increase in cadherin levels was also observed with FSH and T treatment (data not shown).

A 2-day incubation period was then used to investigate the

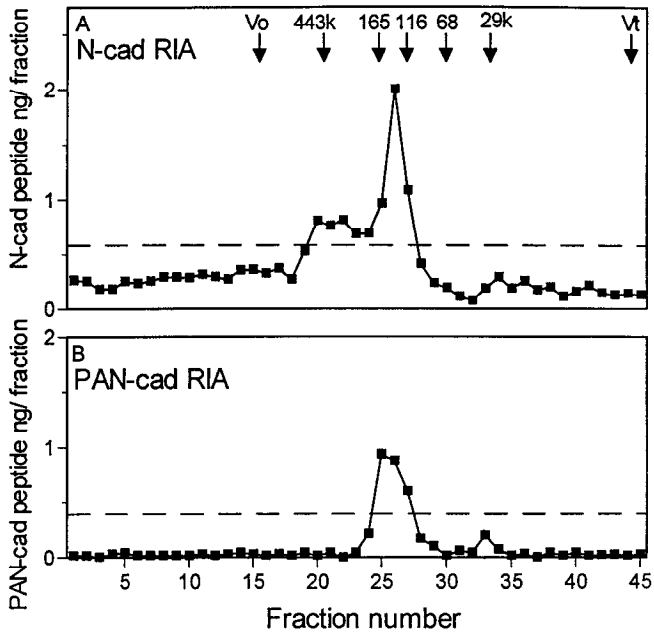

FIG. 1. Profile of *N*-cadherin (A) and PAN-cadherin (B) immunoactivity in rat testis after gel permeation chromatography (Superdex HR200). The positions of mol wt markers used for column calibration are indicated by *arrows* (void volume, V_0; apoferritin, 443K; IgG, 165K; β-galactosidase, 116K; serum albumin, 68K; carbonic anhydrase, 29K; total volume of column, V_t). *Dashed line* represents the sensitivity of the assay.

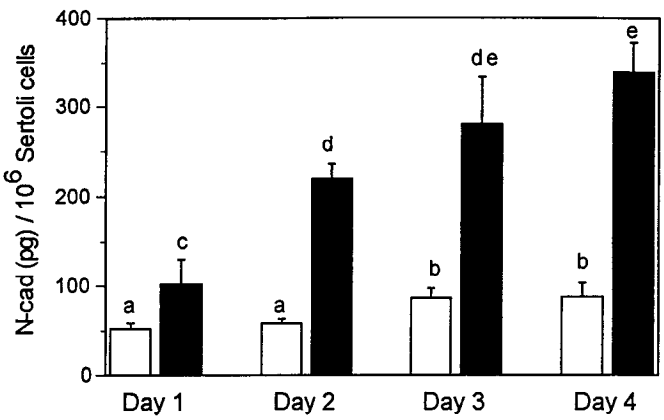

FIG. 2. N-Cadherin levels in Sertoli cells after 1–4 days in culture, as determined by the N-cadherin RIA. Sertoli cells were cultured for 1, 2, 3, and 4 days with medium alone as a control (□) or with FSH (1 μg/ml) and T (28 ng/ml) in combination (■). Each *column* represents the mean ± SD of triplicate estimations. *Columns with different letters* are significantly ($P < 0.05$) different. A representative experiment is presented.

effect of increasing concentrations of FSH and T on N-cadherin levels in Sertoli cell cultures (Figs. 3 and 4, A–C). FSH (0.25–1 μg/ml) and T (7–28 ng/ml) did not affect N-cadherin production [Fig. 4A; only data for FSH (1 μg/ml) and T (28 ng/ml) shown]; however, a 2-fold stimulation was seen with 100 ng/ml T. Graded doses of FSH (0.025–1 μg/ml) in the presence of a fixed dose of T (28 ng/ml; Fig. 3) showed a dose-related increase in N-cadherin levels, with an ED_{50} of 0.12 μg/ml. Graded doses of T (0–100 ng/ml) with FSH (1 μg/ml) also showed a dose-related increase, with an ED_{50} of

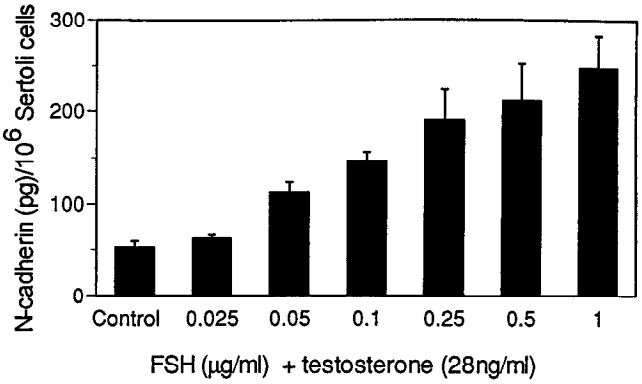

FIG. 3. Effects of FSH and T on N-cadherin levels in Sertoli cell only cultures. Sertoli cells were treated with graded doses of FSH (0.025–1 μg/ml) in the presence of T (28 ng/ml), solubilized, and assayed in the N-cadherin RIA. *Columns* represent the mean ± SD of quadruplicate estimations. A representative experiment is presented.

14 ng/ml. The increase in N-cadherin levels was not a reflection of an increase in cell protein content, as cell protein levels remained unchanged after combined FSH/T treatments.

Round spermatid binding to Sertoli cells in vitro

The round spermatid-Sertoli cell *in vitro* adhesion assay was used to determine the effects of FSH and T treatment. Cocultures were incubated with FSH (1 μg/ml), T (28 ng/ml), and increasing concentrations of either FSH or T in the presence of fixed concentrations of T and FSH. FSH or T produced no effect on round spermatid binding when expressed in terms of the number of round spermatids per 10,000 μm^2 Sertoli cell culture surface area (Fig. 4D). However, a significant ($P < 0.05$) 5-fold increase in the binding of round spermatids was observed with FSH (1 μg/ml) in the presence of T (28 ng/ml; Fig. 4E). In addition, a T-dependent dose-response effect on round spermatid binding was observed with constant FSH (1 μg/ml), with a maximum response at 28 ng/ml T (Fig. 4F).

Production of N-cadherin by Sertoli cell-round spermatid cocultures

Sertoli cell-round spermatid cocultures were treated with FSH in the presence of increasing concentrations of T (0–100 ng/ml; Fig. 5). N-Cadherin levels increased 4.6-fold, similar to that seen (3.7-fold) in Sertoli cell cultures alone (Fig. 3, B and C). Of interest, T (28 ng/ml) in coculture produced a significant increase in N-cadherin not seen previously with Sertoli cells alone at this dose of T (Fig. 4A).

Effect of N-cadherin immunoneutralization on the binding of round spermatids to Sertoli cells in vitro

Sertoli cell-round spermatid cocultures were incubated with N-cadherin antiserum to immunoneutralize any N-cadherin-mediated binding of round spermatids to Sertoli cells. A 65% suppression of spermatid binding was observed in the presence of N-cadherin antiserum (Fig. 6). This N-cadherin immunoneutralization effect could be totally abolished by preincubation of the N-cadherin antiserum with N-cadherin

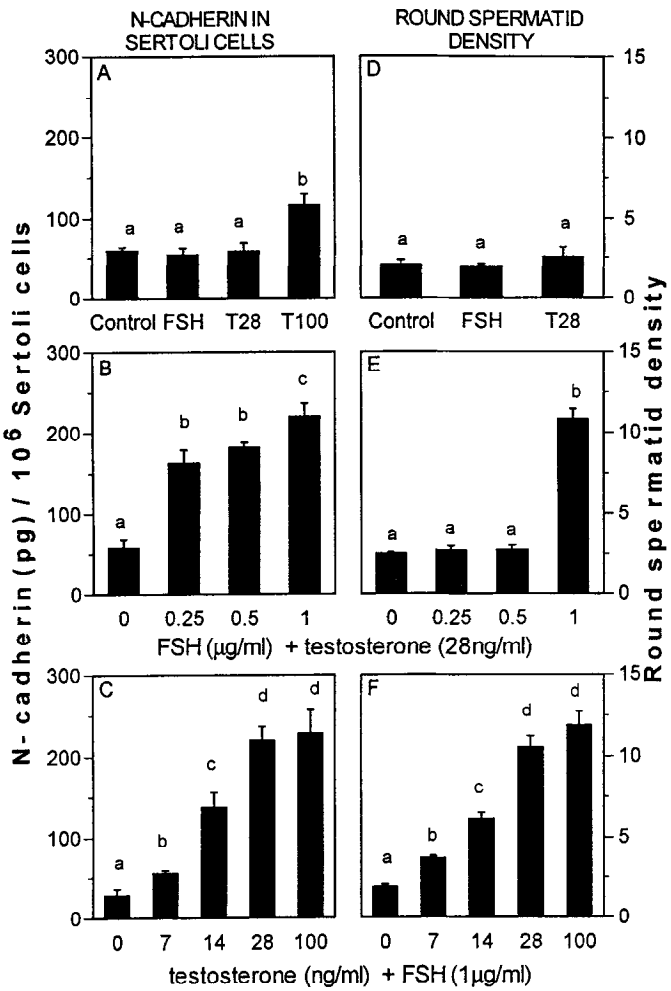

FIG. 4. Effects of FSH and T on N-cadherin levels in Sertoli cell only cultures (A–C) and round spermatid binding to Sertoli cells in coculture (D–F). Sertoli cells were treated with FSH (1 μg/ml) or T (28 and 100 ng/ml) alone (A), increasing doses of FSH (0–1 μg/ml) in the presence of T (28 ng/ml; B), and increasing doses of T (0–100 ng/ml) in the presence of FSH (1 μg/ml; C) for 48 h, solubilized, and assayed in the N-cadherin RIA. Cocultures were treated with this hormonal regimen (D–F) before quantitation of round spermatid density. For A–C, *columns* represent the mean ± SD of triplicate estimations. For D–F, each *column* represents the mean ± SD of three experiments. *Columns with different letters* are significantly ($P < 0.05$) different.

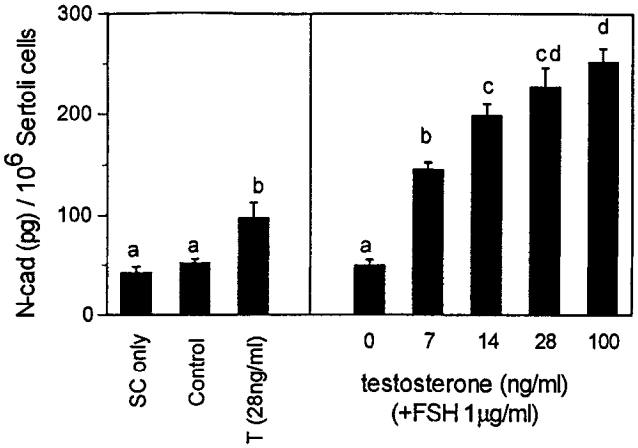

FIG. 5. N-Cadherin levels in Sertoli cell-round spermatid cocultures, as determined by the N-cadherin RIA. Cocultures were treated with T alone (28 ng/ml) or with increasing concentrations of T (0–100 ng/ml) in the presence of FSH (1 μg/ml). *Columns* represent the mean ± SD of triplicate estimations. Different letters denote significant ($P < 0.05$) differences between treatment groups. A representative experiment is presented.

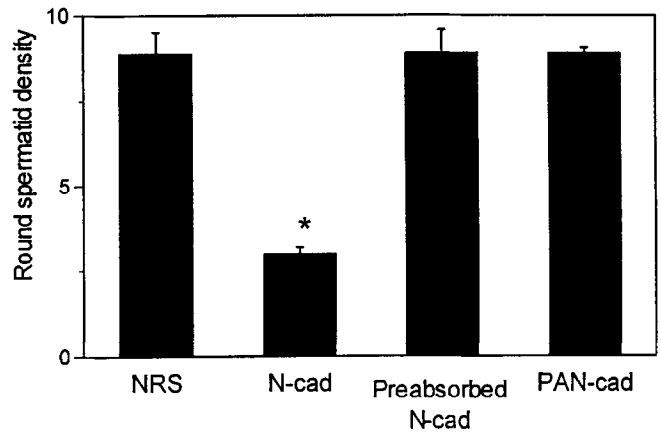

FIG. 6. Round spermatid density after a 48-h incubation in coculture with Sertoli cells. Treatments consisted of cocultures incubated with NRS (1:100), N-cadherin antiserum (N-cadherin 6; 1:100), N-cadherin antiserum preabsorbed with its immunizing peptide, and PAN-cadherin antiserum (PAN-cad; 1:100). Round spermatid density = number of round spermatids/10,000 μm^2 Sertoli cell culture surface area. Each *column* represents the mean ± SD of three experiments. *, $P < 0.0001$.

peptide [N-cadherin-(238–252)]. In contrast, PAN-cadherin antiserum, which is directed toward the cytoplasmic domain of cadherin and, therefore, not expected to have an effect, showed no immunoneutralization.

Discussion

This study demonstrates a role for N-cadherin in the binding of round spermatids to Sertoli cells *in vitro* and the synergistic actions of T and FSH in the parallel induction of both N-cadherin expression and spermatid binding. The active involvement of N-cadherin in round spermatid-Sertoli cell adhesion *in vitro* was shown by the marked reduction in spermatid adhesion after immunoneutralization of N-cadherin. Overall, the data strengthen the proposition that N-cadherin is a candidate for one of the factors subserving the androgen-dependent process of round to elongated spermatid maturation *in vivo*.

We have shown that N-cadherin immunoactivity is produced by Sertoli cells in culture and that N-cadherin levels are stimulated in a dose-dependent manner by T in the presence of FSH. Similar findings were obtained using the PAN-cadherin RIA directed to the intracellular domain of the N-cadherin molecule. We believe that T exerts a direct action on the Sertoli cell, although the influence of low contaminating levels of peritubular cells (<5%) in our cultures, which may promote an indirect T action, cannot be excluded.

The specificity of both cadherin RIAs was underscored by their detection of a 138K immunoreactive peak after gel permeation chromatography of solubilized rat testis, a result consistent with our Western blotting studies and other stud-

ies using identical (16, 31) or very similar peptides (15, 19, 39) to generate antibodies. A second higher mol wt peak of approximately 250K was also detected with the N-cadherin antiserum, which could represent N-cadherin bound to an intracellular protein(s), such as the catenins (40); however, this was not further investigated. A 68K band detected by Western blotting presumably represents a proteolytic product.

Previous investigators have demonstrated mRNA for N-cadherin in the murine testis (13, 16, 20, 21) and the presence of N-cadherin in Sertoli cells and germ cells using immunocytochemistry (16, 39); however, the current study is the first to quantify the expression of N-cadherin by RIA and to apply this procedure to studies of the hormonal regulation of N-cadherin *in vitro*. Cadherins have been found to be hormonally regulated in both male and female reproductive organs, although not in Sertoli cell cultures. Estradiol treatment stimulated mRNA levels for both the E (epithelial)-cadherin and N-cadherin subtypes in ovarian granulosa cells (41, 42). N-cadherin mRNA in the immature mouse testis appeared to be stimulated only by estradiol as dihydrotestosterone was ineffective, as was T when its aromatization to estradiol was inhibited (21). Epithelial cadherin has also been found to be androgen dependent in the epididymis (13).

We have now shown that T and FSH stimulate N-cadherin production in cultures of immature rat Sertoli cells. Maximal stimulation of Sertoli cell N-cadherin content was observed with 28 ng/ml T plus 1 μg/ml FSH. A T concentration of 100 ng/ml, which approximates the normal level of intratesticular T in adult rats, resulted in a doubling of N-cadherin immunoactivity. Production of N-cadherin in the Sertoli cell culture was hormonally stimulated by T concentrations greater than 7 ng/ml, well below the normal intratesticular T concentration (6). Responsiveness to such low T concentrations compared to normal *in vivo* levels is consistent with previous studies in T-suppressed rats, in which the complete restoration of elongated spermatid number, and therefore presumably spermatid adhesion, was seen when T levels exceeded 12–14 ng/ml (5, 6, 43). Using this *in vivo* model, where FSH levels are only slightly lowered, premature sloughing of round spermatids from the seminiferous epithelium occurs (5, 7) when intratesticular T levels are suppressed below 3–4 ng/ml (6, 7). We speculate that this sloughing may at least in part be due to the absence of N-cadherin at the intercellular junctions.

A similar increase in and hormonal dependency of N-cadherin were observed when Sertoli cells were cocultured with a purified preparation of round spermatids. The binding of round spermatids to Sertoli cells *in vitro* showed the same pattern of hormonal dependency as the expression of N-cadherin, with a 5-fold increase in spermatid binding obtained with combined FSH and T treatment. Provided that FSH was also present, spermatid binding showed clear T dose dependency, with a maximal degree of binding occurring at 28 ng/ml. A similar pattern of hormone dependency for spermatid binding *in vitro* has been reported previously (11).

The addition of N-cadherin antiserum to the coculture reduced spermatid binding by 65%. The specificity of this immunoneutralization was demonstrated by 1) the lack of effect of the N-cadherin antiserum after preabsorption with the immunizing peptide, and 2) the lack of effect of both NRS and the PAN-cadherin antiserum directed toward the cytoplasmic domain. These results argue strongly that N-cadherin is involved in the intercellular junction between Sertoli cells and round spermatids *in vitro* and support previous evidence implicating N-cadherin in binding between rat Sertoli and spermatogenic cells (16).

Previous morphological studies have demonstrated that FSH and T play a role in the binding of round spermatids to Sertoli cells *in vitro* (11, 22), possibly by enabling an organization of the cytoskeletal elements involved in the junctional complex (44, 45). The current study supports a role for FSH in Sertoli cell structure and binding function by demonstrating that T has a positive effect on round spermatid binding to Sertoli cells only when FSH is present. Limited binding was observed at lower FSH concentrations even though N-cadherin levels were shown by RIA to have increased significantly. We propose that this effect may be due to the higher FSH level required to induce binding competency of the Sertoli cell. Recent experiments (44, 45) also revealed that FSH is important *in vivo* for the peripheral distribution of actin and vinculin, and the formation of structurally intact ES junctions. An alternative explanation for the observation that maximum N-cadherin production occurs at concentrations of T and FSH not associated with maximum Sertoli cell-round spermatid binding is that other adhesion factors are involved.

Round spermatid binding to Sertoli cells was assessed morphologically and necessitated the assumption that round spermatids observed after vigorous washing were actually involved in binding interactions with Sertoli cells rather than undergoing Sertoli cell-mediated phagocytosis (46). This point was investigated by Cameron and Muffly (11), who found that phagocytosis did not occur using a similar spermatid adhesion system and that normal ES junctions developed between step 8–9 spermatids and Sertoli cells. No distinction has been made in the current study regarding the types of junctions formed between Sertoli cells and round spermatids. The established ES junction is highly resistant to aggressive washing and fixation procedures (11), whereas the desmosomal junctions associated with early round spermatid forms are much weaker. The washing conditions in this study were carefully controlled and vigorous; however, the observed germ cell binding may not be attributed solely to ES-mediated junctions, as the round spermatid preparation included those in steps 1–9.

The data are in concordance with the hypothesis that intercellular binding between Sertoli cells and round spermatids is stimulated by T and, in particular, via its effect on Sertoli cell surface adhesion molecules (N-cadherin). However, even though the use of N-cadherin antiserum in immunoneutralization studies resulted in significantly reduced round spermatid binding to Sertoli cells, it did not eliminate binding altogether. This indicates that additional cell adhesion molecules may be involved in the Sertoli cell-round spermatid interaction, such as *N*-CAM (12) or the β_1-integrin subunit (14, 17), both of which have been localized to the seminiferous epithelium in the rat.

In conclusion, this study demonstrates that the binding of

round spermatids to Sertoli cells and the production of Sertoli cell N-cadherin are stimulated in a concentration-dependent manner by T, but only in the presence of FSH. This is in agreement with the hypothesis that round spermatid adhesion to Sertoli cells is severely disrupted by reduced testicular T levels, thereby causing the loss of round spermatids from the seminiferous epithelium. The marked reduction in round spermatid binding by immunoneutralization with N-cadherin strongly supports a role for this molecule in the intercellular binding between round spermatids and Sertoli cells.

Acknowledgments

The authors gratefully acknowledge Liza O'Donnell, Dr. Nigel Wreford, and Dr. Don Cameron for helpful discussions, and Sarah Meachem for expert technical assistance.

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