Thyroid Hormone Down-Regulates Neural Cell Adhesion Molecule Expression and Affects Attachment of Gonocytes in Sertoli Cell-Gonocyte Cocultures*

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ABSTRACT

Contact-mediated interactions between Sertoli cells and gonocytes are important for testicular development. Specifically, down-regulation of neural cell adhesion molecule (NCAM)-based intercellular adhesion during postnatal maturation is likely to be important for appropriate differentiation of testicular cells. Besides NCAM, P-cadherin is also present in neonatal testicular cords, at least in mice, and seems to disappear from the seminiferous epithelium after the first postnatal week. Another factor known to be important in regulating development of the neonatal testis is thyroid hormone (T3). T3 is involved in control of Sertoli cell proliferation and differentiation. Therefore, we examined the effect(s) of T3 on adhesive factors found within the testis using Sertoli cells and gonocytes isolated from neonates and maintained in coculture.

T3 (100 nM) down-regulated NCAM expression in vitro, as assessed by Western blotting and immunofluorescent staining. This contrasted with the continued expression of NCAM in cultures without added T3 but mimicked the disappearance of NCAM from the neonatal rat testis in vivo. In addition, Western analysis confirmed that P-cadherin is highly expressed in the developing rat testes, as it is in those of mice. We found that P-cadherin is strongly expressed in gonocytes and weakly expressed in Sertoli cells. Moreover, unlike NCAM, P-cadherin expression diminishes with time in vitro in the absence of added hormones. In parallel with our observations for NCAM, expression of P-cadherin was also apparently decreased by T3 (100 nM).

Subsequent quantitative analyses of cultures exposed to a range of T3 levels (0.1–100 nM) indicated that T3 causes detachment of many gonocytes in a dose- and time-dependent manner (approximately 80% detached at 100 nM). In addition, Western blotting indicated that lower concentrations of T3 down-regulate NCAM but not P-cadherin. From this we conclude that the apparent decrease in P-cadherin induced by 100 nM T3 and detected on Western blots reflects loss of gonocytes. In contrast, even low levels of T3 appear to down-regulate NCAM production before any significant detachment of gonocytes. Finally, low levels of T3 that did not affect numbers of adherent Sertoli cells nevertheless caused detachment of gonocytes. Thus, our observations identify T3 as a regulator of NCAM expression in neonatal testicular cells and as a modifier of gonocyte/Sertoli cell adhesion in vitro. (Endocrinology 141: 1633–1641, 2000)

FERTILITY IN ADULT male rats is dependent on the production of mature spermatogenic cells. These cells differentiate from spermatogonia that arise during development from postnatal gonocytes. Little is known about what regulates the maturation of gonocytes, although a close physical and functional relationship exists between germ and Sertoli cells (1), suggesting that neonatal development of the two populations is interdependent. For example, at birth, gonocytes are mitotically quiescent; but shortly after birth, these cells resume mitosis and begin migrating toward the basal lamina (2). At the time that gonocytes divide and migrate, some Sertoli cells stop dividing and, by postnatal day 15, virtually all Sertoli cells become quiescent, to form the terminally differentiated population of adult cells. Thus, differentiation of Sertoli cells and postnatal germ cell matura-
propionate expression of NCAM may be particularly critical during neonatal testicular development. In addition, other adhesion molecules, including P-cadherin (11–15), have been detected in developing testes, although we know little about the function and regulation of these factors. Hence, we have begun to examine more closely the roles played by both NCAM and other adhesive molecules within the developing testis.

Substantial evidence, obtained both in vivo and in vitro, indicates that T3 is an important modulator of testicular development. For example, experimental hypothyroidism leads to a prolonged period of Sertoli cell proliferation and a delay in differentiation of these cells, whereas hyperthyroidism causes a shortened period of Sertoli cell proliferation and accelerated Sertoli cell differentiation (16–23). In addition, when FSH-stimulated Sertoli cells are exposed to T3 in vitro, their rate of proliferation is reduced, and a number of markers of Sertoli cell differentiation appear (16). Thus, T3 is believed to be an important physiological stimulus that causes Sertoli cells to cease mitotic activity and differentiate. Interestingly, T3 may also regulate germ cell development, because gonocytes persist longer than normal in hypothyroid rats (22). This is likely to be mediated via an effect because gonocytes persist longer than normal in hypothyroid- and accelerated Sertoli cell differentiation (16 –23). In addition, experimental hypothyroidism causes Sertoli cells to cease mitotic activity and differentiate.

Materials and Methods

Animals

Male rat pups were obtained by controlled breeding of adult CD Sprague Dawley rats (Charles River Laboratories, Inc. Breeding Labs, Kingston, RI). All animals were maintained and fed water and lab chow ad libitum in an environmentally controlled facility at Temple University School of Medicine, in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Gonocyte-Sertoli cell cocultures

Cocultures of gonocytes and Sertoli cells were prepared as previously described (5). Cultures were maintained in hormone- and serum-free Eagle’s D-Valine MEM (Life Technologies) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 3 mM sodium lactate, 5 μg/ml transferrin, and 50 ng/ml retinol in a water-saturated environment of 95% air, 5% CO2 at 37 C for up to 15 days. One day after plating, appropriate chambers and dishes were treated with 0, 0.1, 1, 10, or 100 nM T3 (Calbiochem, San Diego, CA) in hormone- and serum-free Eagle’s D-Valine MEM. T3 was prepared fresh and replenished in chambers or dishes every 24 h.

Preparation of protein samples

Tissue samples. Adult rats and rat pups (1, 3, 5, 10, 15, or 23 days old) were killed by CO2 asphyxiation; and testes were immediately removed, decapsulated, snap-frozen in liquid nitrogen, and stored at −70 C. Protein samples for Western blotting were prepared with a modification of the method described by Rougon et al. (29). Briefly, frozen tissues were thawed; hand-homogenized on ice in a lysis buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 5 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin; followed by incubation at 4 C for 60 min. Tissue lysates were then centrifuged at 14,000 × g at 4 C for 30 min, and supernatants were collected into fresh tubes. Protein concentrations of the supernatants were determined using a bicinechonic protein assay kit (Pierce Chemical Co., Rockford, IL).

Cells. Protein samples were prepared from cocultures after 1, 2, 4, 5, 10, or 15 days in culture. After rinsing gently with fresh medium, adherent cells were harvested with a cell scraper (Fisher Scientific, Pittsburgh, PA), subsequently pelleted, and stored at −70 C until preparation of proteins, as described above for tissues.

Western Blotting

Proteins were equally loaded on each gel (50–100 μg total protein/ lane) and separated by electrophoresis on a 5 or 7.5% SDS-polyacrylamide gel, then electrophoretically transferred to a nitrocellulose membrane. After incubation in 5% dried nonfat milk powder in Tris buffered saline with 0.1% Tween-20 at 4 C overnight, membranes were probed with primary antibody for 60 min at room temperature (RT). After four washes of at least 10 min each in Tris buffered saline with 0.1% Tween-20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody [goat antirabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for RO49 or rabbit antigoat IgG (H+L; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for anti-P-cadherin] for 30 min at RT. Antibodies specifically bound to the membrane were visualized by chemiluminescence with a SuperSignal kit (Pierce Chemical Co.), applied according to the manufacturer’s directions. To verify that lanes had been equally loaded with protein, some membranes were stripped, using the Chemicon Re-Blot stripping procedure (Chemicon, Temecula, CA), and reprobed with a mouse monoclonal IgG specific for β-tubulin (Roche Molecular Biochemicals, Indianapolis, IN). Analyses of P-cadherin and NCAM were each performed a minimum of three times, to ensure that results were reproducible. Densitometric data were obtained from Western blots using the Bioquant (R & M Biometrics, Inc., Nashville, TN) software suite (Version 3.00.6). Data were normalized to β-tubulin and expressed as a percentage of the untreated control (mean ± SE).

Northern blotting

Total RNA was obtained from testis tissue or cocultures, using the QIAGEN RNasey kit according to the manufacturer’s instructions (QIAGEN, Valencia, CA). Samples were collected and stored as for protein samples, and 20 μg RNA/lane was run on a 1% agarose gel
containing formaldehyde, then transferred overnight, in 20 × SSC, to nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Membranes were prehybridized for 30 min in Rapid-Hyb (Amersham Pharmacia Biotech); and boiled [32P]-labeled P-cadherin complementary DNA (cDNA) probe, a gift of M. Takeichi [clone p28, described by Nose et al. (30) and Cyr et al. (12)] was then added in Rapid-Hyb. This cDNA probe was labeled using the Rediprime Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. After hybridization, membranes were washed in decreasing concentrations of SSC, 0.1% SDS before being analyzed autoradiographically using Biomax MS film and intensifying screens (Eastman Kodak Co., Rochester, NY).

**Immunofluorescence**

Gonocyte/Sertoli cell cocultures were fixed in 2% paraformaldehyde in PBS (pH 7.2) for 20 min at RT, followed by washes in PBS; and immunolocalization was performed as previously described (8). Chambers were blocked in 10% normal donkey serum (for P-cadherin) or 10% normal goat serum (for NCAM) at RT for 60 min and then incubated with primary antibody (P-cadherin, 1:10 in 10% normal donkey serum; RO49, 1:100 in 10% normal goat serum) overnight at 4 C. Control chambers were incubated either in preimmune serum or without primary antibody. After extensive washing in PBS, chambers were incubated in the appropriate rhodamine conjugated secondary antibody for 2 h at RT (P-cadherin: a 1:500 dilution of donkey antigoat IgG; RO49: a 1:1000 dilution of goat antirabbit IgG [both, Jackson ImmunoResearch Laboratories, Inc.]). After rinsing, slides were mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA) and then either viewed and photographed with differential interference contrast (DIC) and epifluorescence optics on an Orthoplan 2 microscope (Leitz, Rockleigh, NJ) or images were captured using an Eclipse E800 microscope (Nikon Instrument group, Melville, NY) attached to a DEI-750 CE digital video camera and software (Optronics, Boston, MA).

**Quantitative analysis of cell number after T3 treatment**

To quantify the numbers of cells in T3-treated and control groups, triplicate chambers in each group were analyzed as previously described (8). For quantitative data obtained as described above, one-way ANOVA was used to determine whether differences existed among the mean values for control and treatment groups, and these differences were subsequently located with a Newman-Keuls test.

**Results**

**Effect of T3 on expression of NCAM**

We previously found that NCAM expression remains high in gonocyte/Sertoli cell cocultures prepared on postnatal day 5 and maintained for up to 15 days in vitro, in contrast to down-regulation of NCAM with increasing age in vivo (8, 9). In the present study, we incubated cocultures with 100 nm T3 or with vehicle for 5, 10, or 15 days and examined NCAM production by applying Western analysis to protein samples isolated from these cultures. After exposure to T3 for 5 days, there was a marked decrease in NCAM, compared with controls (Fig. 1A). Moreover, after longer culture, for either 10 or 15 days, we detected negligible NCAM signal in samples from T3-treated cultures, whereas NCAM in vehicle-treated controls remained high at both times. Importantly, even after prolonged exposure to T3, an essentially confluent monolayer of Sertoli cells remained in the dishes, though few gonocytes were observed (Fig. 1B). To evaluate further the effect of T3 on NCAM in cocultured cells, we also used immunofluorescence to visualize NCAM in cocultures prepared from either day-1 or day-5 rat testes and maintained for 5 days with or without T3 (100 nm). In both cases, addition of hormone caused an obvious decrease in NCAM signal, compared with the strong fluorescence seen in untreated controls (Fig. 2). Specifically, the localization of NCAM to cell-cell boundaries was greatly decreased by exposure to T3, although some diffuse, cytoplasmic fluorescence was still present in the hormone-treated cells. In addition, it seemed that there were fewer attached gonocytes and more detached, floating cells in the T3-treated chambers, compared with vehicle-treated controls.

**p-cadherin expression in vivo and in vitro**

To assess p-cadherin expression in the rat testis during postnatal development, Western and Northern blotting analyses were applied to protein, and total RNA was obtained from testes on days 1, 3, 5, 10, 15, 23, or from testes of adults (Fig. 3). p-cadherin protein levels were essentially stable through day 10, dropped slightly by day 15, and then became low to absent in testes of adults. Expression of p-cadherin mRNA also tended to decrease during development, although a weak band could still be seen in samples from adults. Western analysis was also applied to
protein samples obtained from neonatal cells cocultured for 1, 2, or 4 days (Fig. 4A). A single strong band indicative of P-cadherin was apparent in control cultures after 24 or 48 h. However, essentially no P-cadherin was detected in protein samples from cocultures maintained for 4 days in vitro (Fig. 4A).

When P-cadherin was immunolocalized in cocultures prepared from testes of newborn pups, fluorescent signal was seen in both Sertoli cells and gonocytes (Fig. 4B). However, germ cells displayed noticeably stronger fluorescence than did Sertoli cells, and much of this signal was cytoplasmic, with only a few restricted areas with signal apparently at cell surfaces.
We also used Western analysis to determine whether 100 nM T$_3$ affects production of P-cadherin in cocultured Sertoli cells and gonocytes. We found that it was unchanged after 1 day of treatment with T$_3$, but, by day 2 in vitro, there was an obvious decrease in P-cadherin signal in hormone-treated cultures, compared with controls (Fig. 4A). It was again noted that there were fewer attached gonocytes and more detached, floating cells in the T$_3$-treated chambers, compared with vehicle-treated controls.

**Effect of T$_3$ on numbers of cocultured Sertoli cells and gonocytes**

Qualitative changes in the numbers of adherent vs. floating cells in cocultures were noted when cells were exposed to 100 nM T$_3$. Therefore, we examined the impact of a range of T$_3$ concentrations (0.1–100 nM) on the numbers of both gonocytes and Sertoli cells remaining adherent with increasing time in vitro. Cocultures were prepared from day-1 testes and incubated with 0, 0.1, 1, 10, or 100 nM T$_3$, each for 24, 48, or 96 h. Each chamber was then fixed and quantified to determine the numbers of Sertoli cells and gonocytes remaining, as outlined in Materials and Methods. After 24 h, the number of gonocytes remaining in the monolayers was significantly reduced ($P < 0.05$) in the presence of 100 nM T$_3$ but not at lower doses (Fig. 5A), whereas no change was found in Sertoli cell numbers in any of the groups after 24 h of T$_3$ treatment. After 48 h, the numbers of gonocytes were significantly reduced by treatment with 1, 10, or 100 nM T$_3$, whereas Sertoli cell numbers again remained unchanged in all groups (Fig. 5B). After 96 h, the numbers of gonocytes remaining in all of the treated chambers (0.1–100 nM T$_3$) were lowered substantially, compared with controls (Fig. 5C). We again detected no change in Sertoli cell numbers after exposure to 0.1–10 nM T$_3$. However, by 96 h of treatment with 100 nM T$_3$, there was about a 30% decrease in the numbers...
of Sertoli cells, compared with control cultures. Thus, exposure of cocultures to 0.1–10 nM T₃, for 24–96 h, causes loss of gonocytes that is not accompanied by a decrease in Sertoli cells.

Effect of 0.1–10 nM T₃ on P-cadherin and NCAM

To determine whether a T₃-induced decrease in NCAM or P-cadherin preceded detachment of gonocytes, we extended our Western analyses to examine cultures exposed to lower levels of hormone. Doses were chosen that did not cause a decrease in either gonocyte or Sertoli cell numbers, as determined in the experiments described above.

NCAM. Western blotting was applied to samples obtained from cocultures incubated for 24–48 h with 0, 0.1, or 10 nM T₃. In each of five separate experiments for which equal loading of protein across lanes was verified by reprobing the blot for β-tubulin. This immunoblot is representative of five replicates, in each of which a similar noticeable decline in NCAM production was detected at either a T₃ dosage or exposure time not found to cause detachment of gonocytes. B, Western blots (as above) were quantified, and the results were expressed as a percentage of the untreated control (mean ± SE of five experiments).

P-cadherin. Protein samples obtained from cultures exposed to 0, 0.1, or 10 nM T₃ for 24–48 h were also immunoblotted for P-cadherin, with a protocol identical to that performed in earlier analyses. The result of several experiments indicates that P-cadherin production is unaffected by the presence of T₃ (see Fig. 8A for representative Western blot). Similarly, when total RNA obtained from similarly treated cultures was probed for the presence of P-cadherin mRNA, we detected no effect of 0.1–10 nM T₃ on its expression (Fig. 8B).

Discussion

Our findings demonstrate, for the first time, that T₃ regulates production of NCAM in cells isolated from the neonatal seminiferous cord and maintained in vitro. Moreover, T₃-induced down-regulation of NCAM may precede detachment of some gonocytes from the underlying monolayer of Sertoli cells, which occurs as early as 24 h after the start of treatment and is enhanced in a dose- and time-responsive manner. In addition, at doses of T₃ below 100 nM, detachment of gonocytes occurs without any accompanying changes in
the number of adherent Sertoli cells. Thus, the most striking effects of treating Sertoli cell-gonocyte cocultures with T3 are NCAM down-regulation and impairment of the ability of many gonocytes to adhere to Sertoli cells. In this regard, it is important to note that diminished NCAM signal in Western blots from T3-treated cultures does not merely reflect loss of NCAM-positive gonocytes, because these cells comprise only about 2% of the cells in the cultures (8). Moreover, because (in some experiments) Western blotting revealed a T3-induced decrease in NCAM production that preceded any significant loss of gonocytes from the cultures, subsequent detachment of these cells is potentially the result of T3-induced down-regulation of NCAM. This presumably reflects an indirect effect of the hormone on these germ cells via its action on Sertoli cells, because available evidence strongly suggests that the latter are the primary (if not only) T3 targets in the seminiferous epithelium (20, 24–27). Thus, the ability of gonocytes to attach to Sertoli cells, at least in vitro, seems to be altered by exposure of the latter to T3, a hormone well-recognized as a regulator of Sertoli cell differentiation.

Gonocyte survival in vivo, and potentially in vitro, is obviously a result of their ability to avoid apoptosis (32), as well as their expression of appropriate adhesion factors. Thus, it is possible that an apoptotic pathway may be influenced by T3, subsequently affecting gonocyte survival and/or detachment. From our study, this seems unlikely, because we observed no gonocytes displaying morphological evidence of apoptosis, even in cells clearly in the act of rounding up before detachment (not shown). However, this does not rule out the possibility that T3-treatment causes expression of genes, signifying the start of apoptosis. Though not within the scope of the present endeavor, the potential regulation of gonocyte apoptosis by T3 is an important topic and one that we will address in detail in our future work.

Interestingly, even at the highest tested doses of T3, some gonocytes remained adherent to the underlying Sertoli cells, in spite of the virtual absence of NCAM in the cultures. For example, after 4 days of treatment with 100 nM T3, we found that the numbers of gonocytes in treated chambers were about 20% of those seen in controls while an essentially confluent Sertoli cell monolayer was present, in the presence of minimal NCAM expression. This observation raises some interesting questions about the gonocyte population, because those cells that remain adherent after T3 treatment and
Our current findings, indicating a role for T3 in regulation of NCAM expression, correlate well with our understanding of the impact of this hormone on testicular development and with available information on NCAM expression during development. We previously immunolocalized NCAM to Sertoli cell-Sertoli cell and Sertoli cell-germ cell interfaces in neonatal testes in vivo and in vitro, and we also showed that NCAM expression in vivo is increasingly down-regulated as postnatal testicular development proceeds (9). In contrast, during extended coculture of gonocytes and Sertoli cells, we found that the pattern of NCAM expression, over time, did not parallel that seen in vivo. Rather, in these cultures, we noted steady production of NCAM, during 15 days of culture, in the absence of any added hormones or growth factors, implying that exogenous factor(s) might be responsible for the down-regulation of NCAM observed in vivo. Based on data presented here, one factor responsible for down-regulation of NCAM in vivo may be thyroid hormone, a suggestion further supported by data indicating a role for T3 in down-regulating NCAM expression in developing brain (28). In addition, the temporal pattern of expression of testicular T3 receptors in vivo also correlates well with diminishing NCAM production, given that the number of available T3 receptors is high early in postnatal development (24, 27), when down-regulation of NCAM occurs, and then decreases later, after NCAM has essentially disappeared from the seminiferous tubule (9). We already recognize T3 as a hormone of major importance for differentiation of the Sertoli cell population and have, until now, attributed this effect largely to its influence on cell proliferation and apparent exit of Sertoli cells from the cell cycle. Our current findings in vitro raise the possibility that T3 may influence other aspects of Sertoli cell and gonocyte development in the neonate, including (but not limited to) expression of adhesive factors important for interaction of these cells.

It is possible that factors other than NCAM may have a role in contact-mediated cell interactions during neonatal testicular development. Messenger RNA (mRNA) for several members of the cadherin family has been detected at relatively high levels in developing mice (11, 14). In addition, P-cadherin has been immunolocalized to the seminiferous epithelium of newborn mice and, after postnatal day 8, found to be down-regulated in the epithelium and restricted to peritubular cells (13). This raises the possibility that P-cadherin may be important for interaction between Sertoli cells and gonocytes during the first postnatal week, and this prompted us to begin exploring its expression in neonatal testicular cells in vitro. After confirming its expression in vivo to be at high levels in testes during the first 2 postnatal weeks, we immunolocalized P-cadherin in cocultures and found it to be preferentially expressed in gonocytes, with only low- to moderate localization to Sertoli cells. Moreover, its localization is primarily intracellular, with little signal at cell-cell boundaries, implying that P-cadherin may not act as a classical adhesion factor, at least in gonocytes and Sertoli cells under these in vitro conditions. Interestingly, although the qualitative observations made by Lin and DePhilip (13) on P-cadherin localization in testes of newborn mice in vivo were somewhat different from ours, these authors also concluded

Fig. 8. Representative Western (A) and Northern (B) analyses of P-cadherin (P-cad) in protein and total RNA samples isolated from cocultures maintained with levels of T3 found to cause down-regulation of NCAM (Fig. 6). Equal loading of protein across lanes was verified by reprobing Western blots for β-tubulin. In three separate replicates, no changes in either P-cadherin protein or mRNA were detected after exposure to T3.
that P-cadherin does not act as a gonocyte-Sertoli cell adhesion factor in these animals.

When we explored the temporal pattern of P-cadherin production in untreated cocultures, we found that its expression diminishes with increasing time of culture, in direct contrast to the sustained pattern of NCAM production found in similar chambers. Moreover, although inclusion of 100 nM T3 seemed to accelerate the decrease in P-cadherin, as measured by Western analysis, we conclude that this apparent loss of P-cadherin was most likely attributable to detachment of about 80% of the gonocytes after T3-mediated down-regulation of NCAM, because these cells express P-cadherin at extremely high levels, as discussed above. This suggestion is further supported by our finding that P-cadherin protein and mRNA levels remain high after exposure of cocultures to lower doses of T3 that down-regulate NCAM but leave gonocyte numbers unaffected. Thus, T3 does not seem to regulate P-cadherin expression in cultured neonatal testicular cells, at least under the conditions tested thus far.

In summary, our findings provide the first evidence of a role for T3 as a specific regulator of NCAM production by neonatal testicular cells in vitro and suggest that thyroid hormone may play a role in regulation of its expression in vivo. They further suggest that T3, via its action on Sertoli cells, may have a role in determining the ability of some gonocytes to attach to Sertoli cells via non-NCAM mechanisms.

References
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