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Sertoli Cell-Germ Cell Interactions and TGFβ1 Expression and Secretion *in Vitro*

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Transforming growth factor β_1 (TGFβ_1) has been reported to be secreted and to act within the somatic cells of the testis. We examined whether the TGFβ_1 expression is present at mRNA and protein levels in purified rat Sertoli cells (SC), purified pachytene spermatocytes (SPC), and early spermatids (SPT) cultured alone or together. SC expressed a single TGFβ_1 transcript of 2.5 kb, but no TGFβ_1 protein could be detected in SC conditioned medium indicating that, if at all, SC secreted less than 10 pg/10^6 cells/24 h. Neither TGFβ_1 mRNA nor protein could be detected in either SPC or SPT. Coculture of SC with either SPC or SPT resulted in a 2-fold increase of TGFβ_1 mRNA and more importantly in the secretion of TGFβ_1 protein. These findings demonstrate that Sertoli cell-germ cell interactions regulate TGFβ_1 expression and secretion and indicate that TGFβ_1 may be involved in spermatogenesis.

In addition to the hormonal control by Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), it is now obvious that cell-cell interactions play an important role in the regulation of testicular function (1-3). Among factors potentially involved in such regulations, TGFβ_1 is a likely important candidate, since it is involved in the regulation of cell proliferation and differentiation in various tissues and organs, including gonads (3-5). Both TGFβ_1 mRNA and TGFβ-like immunoreactive material are present in the testis of several mammals (for review: 3). In rat, peritubular and Sertoli cells were found to express TGFβ_1 mRNA and secrete TGFβ-like peptides *in vitro* (6). In pig, it has been shown that Leydig and Sertoli cells from immature testis express TGFβ_1 mRNA and protein both *in vivo* and *in vitro* (7). In the adult mouse testis (8), but not in the rat testis (9), a TGFβ_1 transcript of small size was also found in germ cells. By immunohistochemical studies, using different anti-TGFβ_1 antibodies, two groups have also demonstrated the presence of TGFβ_1 in the rat testis, mainly in Leydig cells; a weak signal was observed in Sertoli cells, but not in peritubular cells (10,11). Contradictory results were published for germ cells: whereas in one study (10), TGFβ_1 could be localized in spermatocytes, no such staining was reported in another study (11).

TGFβ_1 is a potent inhibitor of differentiated functions of Leydig cells in several species (12-14). In addition, it exerts marked effects on peritubular cell differentiation (9,15) and modulates some aspects of Sertoli cell function (9,16). Moreover, a recent study has evidenced that isolated testicular cells, including germ cells and Sertoli cells, contain mRNAs for the three types of TGFβ receptors (17).

Although the above data suggest a potential paracrine/autocrine role of TGFβ_1 in the testis, the regulation of its expression in testicular cells, in particular through cell-cell interactions, remains obscure.

The aim of the present work was to investigate TGFβ_1 mRNA expression and protein secretion by isolated rat Sertoli cells and germ cells, using Northern blot and immunoblot analysis. We also investigated the putative involvement of Sertoli cell-germ cell interactions in the paracrine regulation of TGFβ_1 expression.

MATERIALS AND METHODS

Animals

90-day-old and 20-day-old male Sprague-Dawley rats were purchased from Elevage Janvier (Le Genest Saint Isle, France).

Cells and Cultures

Sertoli cell isolation and culture. Sertoli cells were prepared from 20-day-old Sprague-Dawley rat testes, as described previously (18), and cultured at 32°C in a humidified atmosphere of 5% CO_2, 95% air in Ham's F12/Dulbecco's Modified Eagle Medium (DMEM) (1:1 v:v; Gibco BRL, Cergy Pontoise, France), supplemented with gentamicin (50 μg/ml; Gibco BRL), insulin (10μg/ml; Sigma Chemical Co., St

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Louis, MO, USA) and human transferrin (5 μg/ml ; Sigma). The Sertoli cells were seeded at a density of approximately 15×10^6 cells/80 cm^2 Petri dishes (Nunc, Copenhagen, Denmark). After 2-3 days of culture, the Sertoli cell monolayers were exposed to a hypotonic shock (20 mM Tris-HCl buffer solution, pH 7.4) for 2 min, to remove contaminating germ cells (19). The purity of the Sertoli cell culture was then > 98%. One day after the hypotonic shock, the cells were used for coculture experiments (see below) or were incubated for 24 hours in transferrin-free medium alone (Control) or supplemented by lipopolysaccharides (LPS) (20μg/ml; Sigma). At the end of the incubation, Sertoli cell media were recovered, centrifuged, adjusted to pH 3 with acetic acid and frozen at −80°C until Western blot analysis. The Sertoli cell monolayers were then lysed by a denaturing solution of guanidium thiocyanate 4M, sodium citrate 25 mM, sarcosyl 0.5 % and β- mercaptoethanol 0.1 M and stored at −80°C until RNA extraction (20).

Germ cell preparation. Germ cells were prepared from 90-day-old Sprague-Dawley rat testes according to the method of Meistrich *et al.* (21) and then were separated into two cell populations: pachytene spermatocytes (SPC) and early spermatids (SPT), by a centrifugal elutriation (rotor Beckman JE5 driven by a J2-21B centrifuge; Beckman Instruments, Inc, Fullerton, CA, USA) according to a technique described before (22). Cell viability was tested using the trypan blue exclusion test and was found to be at least 95 %. A DNA flow cytometry method (23) was used to estimate the relative DNA content of these three germ cell fractions. The enrichment of the pachytene spermatocyte (4n) and early spermatid (1n) fractions was about 90%. SPC and SPT germ cell fractions were either harvested in a denaturing guanidium thiocyanate solution and stored at −80°C before RNA extraction (20), or cultured to produce conditioned media (see below) or used in coculture experiments (see below).

Preparation of pachytene spermatocyte (SPC) and early spermatid (SPT) conditioned media. After centrifugal elutriation, SPC and SPT were incubated for 18 hours at 32°C in a humidified atmosphere (5% CO_2, 95% air) at the respective concentrations of 2.5×10^6 SPC and 8×10^6 SPT/ml of Ham's F12/DMEM (v/v) supplemented with 6 $mmol.l^{-1}$ of lactate (Boehringer Mannheim, Indianapolis, IN, USA) and 2 $mmol.l^{-1}$ of pyruvate (Sigma) (22). At the end of the incubation, the cell suspensions (cell viability at around 90%) were centrifuged, media were adjusted to pH 3 with acetic acid, and media were frozen at −80°C until Western blot analysis.

Coculture procedure. The day after the hypotonic shock, Sertoli cell monolayers were cultured alone or in the presence of latex beads (3.5×10^8 latex beads/ml; Sigma) or of the respective elutriated populations of germ cells (SPC or SPT), which were added onto Sertoli cell monolayers at the density of 2.5×10^6 /ml for SPC and 8×10^6 /ml for SPT. The day after, media were changed to remove the non-adherent germ cells or latex beads, and then, the cocultures were incubated for an additional 24 hours. Sertoli cell-germ cell coculture media were then recovered, centrifuged, adjusted to pH 3 with acetic acid, and frozen at −80°C until Western blot analysis. Cells were also harvested in a denaturing guanidium thiocyanate solution and stored at −80°C before RNA extraction (20).

Northern Blot Analysis

This procedure was performed as previously described (7). Briefly, total cytoplasmic RNAs (40 μg) were separated by electrophoresis through 1% agarose gel containing 10% formaldehyde and blotted to Hybond-N nylon membrane (Amersham, Les Ulis, France). After transfer, RNAs were cross-linked to the membranes by irradiation for 2 min with UV light and baking at 80°C for 2 h. Prehybridization was performed for 2 h at 42°C in 50% formamide, SSPE [0.75M NaCl, 20mM NaPO4 (pH 7.5), and 1 mM EDTA], 5 × Denhardt's solution (0.1% Ficoll 400, 0.1 % polyvinylpyrolidone, and 0.1 % BSA), 0.1 % sodium dodecyl sulfate (SDS), 10 % dextran sulfate, and 100 μg/ml boiled salmon sperm DNA. Hybridization was carried out overnight at 42°C, in the same prehybridization buffer containing 3×10^6 dpm/ml of ^{32}P-labeled probe, consisting in a 1.05-kilobase (kb) EcoRI fragment of human TGFβ_1 cDNA (gift from Dr. R. Derynck, Genentech, Inc., San Francisco, CA), cut by PvuII in a 0.243-kb fragment. Membranes were washed twice in 2 × SSC (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate) and 0.1 % SDS at room temperature for 15 min each, then twice in 1 × SSC and 0.1% SDS at 65 °C for 15 min each and twice in 0.5 × SSC and 0.1 % SDS at 65°C for 15 min each. Autoradiographs were obtained after one week of exposure at −70°C to Kodak X-AR-K films (Eastman Kodak, Rochester, NY). The blots were analysed by scanning densitometry using an image analyser Samba 2005 (Alcatel, Grenoble, France). The amounts of loading RNA were calculated by scanning 28S negative film.

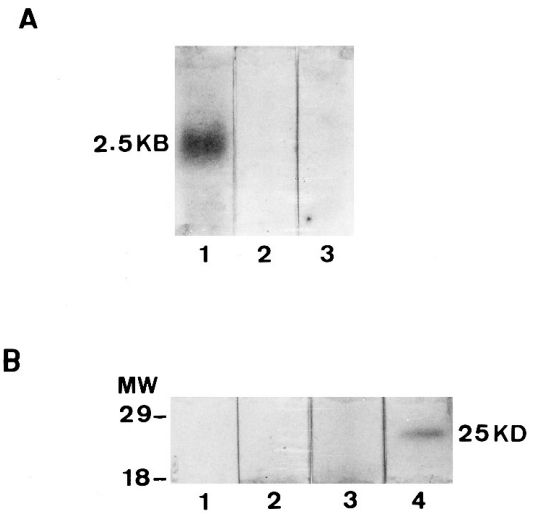

FIG. 1. (**A**) Northern blot analysis of TGFβ_1 mRNA levels in rat Sertoli cells (1), pachytene spermatocytes (2), and early spermatids (3). Forty micrograms of total RNA was hybridized with a radiolabeled TGFβ_1 probe. (**B**) Western blot analysis of 50 ml extracts of conditioned media of rat Sertoli cells (1), pachytene spermatocytes (2), and early spermatids (3) with the specific anti-TGFβ_1 antibody. Lane 4, 2 ng purified TGFβ_1; M.W., molecular weight.

Western Blot Analysis

Western blot analysis was performed as previously described (7). Briefly, 50 ml of the acidified media were lyophilized, then resuspended in 5 ml distilled water and dialysed. After lyophilization, samples were resuspended in Laemmli SDS sample buffer. Then, they were run under non-reducing conditions on a gradient 7.5-15 % SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blots were incubated with a specific anti-TGFβ_1 polyclonal antibody previously characterized (7) and then labeled with radioiodinated protein-A.

Statistical Analysis

Data presented are representative of three or four separate culture/coculture experiments.

RESULTS

Sertoli Cells, But Not Germ Cells Express TGFβ_1 mRNA in Vitro

Using Northern blot analysis, we first investigated the presence of TGFβ_1 mRNA in Sertoli cells and in pachytene spermatocytes and early spermatids (Fig.1A).

After hybridization with a radiolabeled human TGFβ_1 cDNA probe, a single transcript of 2.5 kb was detected in rat Sertoli cells, whereas no such transcript could be visualized, neither in pachytene spermatocytes, nor in early spermatids, even after a long exposure. Similar results were obtained with four different cell preparations.

Sertoli Cells and Germ Cells Do Not Secrete TGFβ_1 in Vitro

The presence of TGFβ_1 in Sertoli cell conditioned media (SCCM), pachytene spermatocyte conditioned media (SPCCM) and early spermatid conditioned media (SPTCM) was then studied by Western blot analysis, using a previously characterized polyclonal anti-TGFβ_1 antibody (Fig.1B). Whereas the antibody recognizes purified TGFβ_1, as evidenced by the visualization of a 25 kDa signal (lane 4), no signal was observed in SCCM (lane 1), SPCCM (lane 2) nor in SPTCM (lane 3).

Sertoli Cell-Germ Cell Coculture Enhances TGFβ_1 mRNA Levels and Induces TGFβ_1 Secretion

To investigate the possible influence of Sertoli cell-germ cell interactions on the expression and secretion of TGFβ_1, enriched preparations of pachytene spermatocytes and early spermatids were cocultured with Sertoli cells. In three independent experiments TGFβ_1 mRNA levels in both coculture conditions, Sertoli + pachytene spermatocytes and Sertoli + spermatids were 2 ± 0.18 and 1.96 ± 0.23 fold higher respectively, than in Sertoli cells cultured alone (Fig.2A). The presence of TGFβ_1 in the conditioned media of Sertoli cells cultured alone or in the presence of germ cells was analysed by Western blot (Fig.2B). As previously seen in Fig.1B, the antibody recognized the purified TGFβ_1 used as positive control (lane 4), whereas no signal was observed in rat Sertoli cell conditioned media (lane 1). In contrast, a 25 kDa signal was visualized in the conditioned media prepared from the cocultures of Sertoli cells with SPC and SPT (lanes 2 and 3, respectively). Under reducing conditions, a 12.5 kDa signal was detected in both these samples (data not shown) as previously described (7). In contrast, none of the known inducers of cytokine production tested, such as LPS (Fig.3), latex beads or phorbol ester PMA (data not shown), were able to modify either TGFβ_1 mRNA level or TGFβ_1 protein secretion by rat Sertoli cells.

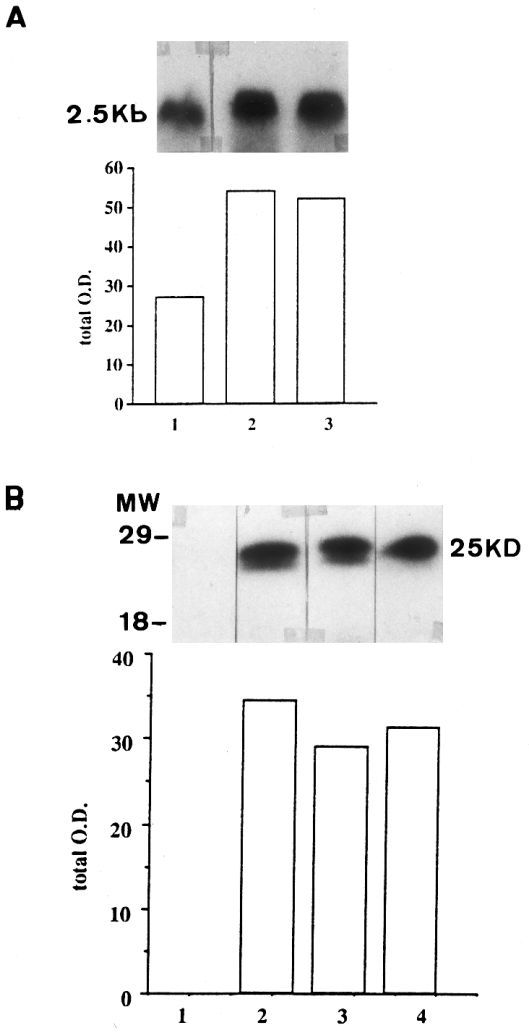

FIG. 2. (**A**) Northern blot analysis of TGFβ_1 mRNA levels in rat Sertoli cells, cultured alone (1) or in the presence of pachytene spermatocytes (2) or early spermatids (3). Forty micrograms of total RNA was hybridized with a radiolabeled TGFβ_1 probe. (**B**) Western blot analysis of conditioned media of rat Sertoli cells cultured alone (1) or in the presence of pachytene spermatocytes (2) or early spermatids (3) with the specific anti-TGFβ_1 antibody. Lane 4, 2 ng purified TGFβ_1; M.W., molecular weight.

DISCUSSION

We first investigated the expression of TGFβ_1 mRNA and protein secretion by isolated rat Sertoli cells and elutriated germ cell fractions, pachytene spermatocytes and early spermatids. We have shown that rat Sertoli cells express the major 2.5 kb TGFβ_1 transcript *in vitro*, but that TGFβ_1 protein could never be demonstrated in rat Sertoli cell conditioned media. Since we used 50 ml of conditioned medium and an antibody which can detect 1 ng of TGF-β (7), the negative result in Fig.1 suggests that the secretion of TGFβ_1 by rat Sertoli cells should be, if any, lower than 10 pg/10^6 cells/24h. These results are in agreement with previous immunohistochemical studies, using the same anti-TGFβ antibody, showing a very faint, if any, immunostaining of immature rat Sertoli cells (11) and with the fact that Western blot analysis of rat immature Sertoli cell extracts (4×10^6 cells) could not detect any TGFβ_1 signal (data not shown). In contrast, immature pig Sertoli cells secrete TGFβ_1 and exhibit strong immunostaining (7). It has been reported that rat Sertoli cells

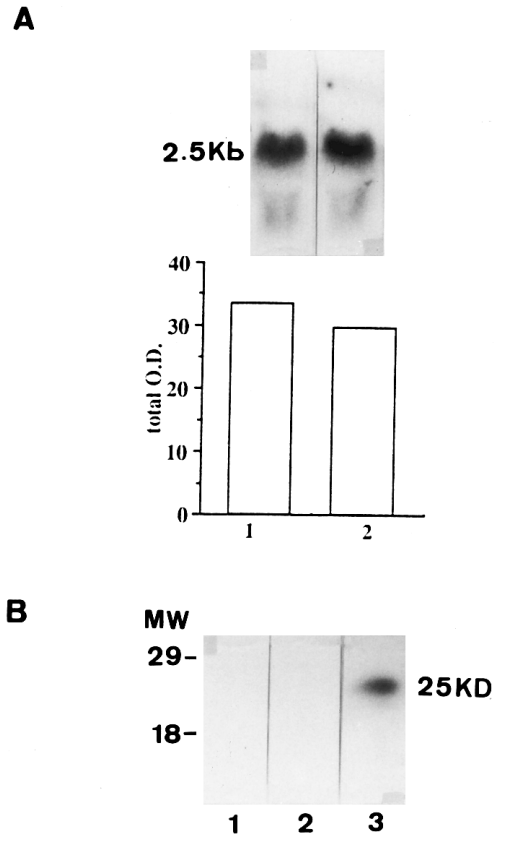

FIG. 3. (**A**) Northern blot analysis of TGFβ_1 mRNA levels in rat Sertoli cells, cultured in control condition (1) or stimulated with 20 μg/ml LPS (2). Data are representative of three separate culture experiments. (**B**) Western blot analysis of conditioned medium of rat Sertoli cells cultured in control condition (1) or stimulated with 20 μg/ml LPS (2) with the specific anti-TGFβ_1 antibody. Lane 3, 2 ng purified TGFβ_1; M.W., molecular weight.

of different ages have the capacity to secrete TGFβ isoforms (6). However, these discrepancies with our findings may result from differences in the epitopes recognized by the antibodies, since these authors used an antibody raised against a synthetic peptide corresponding to the first 30 N-terminal amino acids of TGFβ_1, whose reactivity against TGFβ_3 was not tested. In contrast, we used a polyclonal antibody raised against a synthetic peptide corresponding to amino acids 91 to 103 of mature TGFβ_1, showing no cross-reactivity with TGFβ_2 and TGFβ_3 (7,11).

Our findings also indicate that the expression of TGFβ_1 by isolated germ cells, pachytene spermatocytes and early spermatids, if any, is very low, since neither TGFβ_1 mRNA, nor TGFβ_1 secretion was evidenced. This is consistent with previous observations showing that TGFβ_1 mRNA is most abundant in the immature rat testis and decreases once puberty is initiated (6). This pattern of expression is characteristic of somatic cell expression in the immature testis, followed by dilution of signal due to increased germ cell proliferation.

We have clearly demonstrated that germ cell-Sertoli cell interactions enhance TGFβ_1 mRNA level and initiate, or at least markedly enhance, the secretion of TGFβ_1 by Sertoli cells and/or germ cells *in vitro*. As the anti-TGFβ_1 antibody used in the present study appears to recognize TGFβ_1 with high affinity, but not the two other isoforms found in mammals, TGFβ_2 and TGFβ_3 (7), the presence in immunoblots of a 25 kDa band, which was shifted to 12.5 kDa under reducing conditions, strongly suggests that TGFβ_1 is secreted in conditioned media of Sertoli cell-germ cell cocultures. Whether the enhanced expression and secretion of TGFβ_1 by Sertoli cell-germ cell coculture is due to a stimulatory effect of germ cell on Sertoli cell or of Sertoli cell on germ cell or to both is unknown. However, since previous *in vitro* studies have shown that germ cells are able to influence the production of several Sertoli cell products, through soluble factors and contacts (2,26), we favorised the hypothesis that germ cells positively influence the expression and secretion of TGFβ_1 by Sertoli cells. If this is the case, the stimulatory effect of germ cells on the expression of TGFβ_1 mRNA by Sertoli cells would be higher than that calculated by Northern blot analysis, because in the coculture system, the Sertoli cell mRNA quantity was diluted by that of germ cells.

The apparent discrepancy between the expression of TGFβ_1 mRNA by Sertoli cells and the absence of detectable secretion of TGFβ_1 may be related to the presence in the 5′-untranslated region of TGFβ_1 mRNA of a stem-loop structure which inhibits its translation after binding of a 25 kDa cytosolic protein (24,25). This inhibition is cell specific. A decreased inhibition of translation may also explain the secretion of TGFβ_1 by the coculture of Sertoli cells with germ cells, whereas the TGFβ_1 mRNA only increase 2-fold.

It has been suggested that TGFβ_1 could play a role at the onset of spermatogenesis (6,8,27,28). Such a regulation of TGFβ_1 secretion through interactions between Sertoli cells and germ cells and the fact that Sertoli cells and germ cells contain mRNA for the three types of high affinity TGFβ receptors (17) are in favour of such a hypothesis.

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