—Original Article—

TGF- β 1 System in Leydig Cells. Part II: TGF- β 1 and Progesterone, Through Smad1/5, are Involved in the Hyperplasia/hypertrophy of Leydig Cells

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Abstract. Several reports indicate that transforming growth factor β 1 (TGF- β 1) participates in the regulation of cell cycle progression. In this work, we analyzed the *in vitro* effect of TGF- β 1 on Leydig cell proliferation markers and the *in vivo* effect of this cytokine in Leydig cell hyperplasia/hypertrophy. The *in vitro* effect of TGF- β 1 (1 ng/ml) plus progesterone (10^{-6} M) on purified Leydig cells from 3 week-old mice increased the immunocytochemically detected PCNA and stimulated the phosphorylation of Smad 1/5. Progesterone (10^{-6} M) in the presence or absence of TGF- β 1 diminished the ratio *Bax/Bcl-2*. Morphometric testicular studies of mice treated with progesterone (s.c.) plus TGF- β 1 (intratesticular), showed an increase in interstitial volume and a decrease in tubular volume. Furthermore, the cytoplasmic volume of Leydig cells showed an increment in this experimental group with a diminution in nuclear volume. Thus, it turned out that the administration of progesterone and TGF- β 1 augmented the volume of Leydig cells. These results indicate a clear effect of TGF- β 1 in the hypertrophy/hyperplasia of Leydig cells.

Key words: Leydig, Proliferation markers, TGF- β 1

(J. Reprod. Dev. 56: 400–404, 2010)

TGF- β influences steroidogenesis [1] and also modulates Leydig cell DNA synthesis by regulating Leydig cell proliferation [2]. Moreover, either the absence or overexpression of this factor affects male reproductive function, supporting the participation of TGF- β signaling pathways in the orchestration of testicular development and spermatogenesis [3–5]. The effect of TGF- β 1 is exerted via specific type I and II serine/threonine kinase receptors. Type II receptor (TGF- β RII) transphosphorylates and activates type I receptor (TGF- β RI), which in turn phosphorylates and activates downstream nuclear effectors, named Smads [6]. It has been described that TGF- β 1 activates two distinct type I receptors activin receptor-like kinase 1 (ALK1), which promotes Smad 1/5 phosphorylation, and ALK5, which induces Smad 2/3 phosphorylation. In endothelial cells, endoglin blocks TGF- β -induced growth arrest by promoting ALK-1 signaling, and indirectly reduces ALK-5 signaling leading to cell proliferation [7].

It has been shown that TGF- β and its receptors are altered in pathological conditions such as cancer and hyperplasia of different tissues [8]. Rulli *et al.* [9] have reported that transgenic male mice overexpressing α and β subunits of hCG (hCG+ mice) are infertile and that their reproductive organs show severe alterations. In addition, testicular steroidogenesis is enhanced, showing high levels of circulating testosterone and progesterone. A remarkable feature of

these animals is that chronic hCG hyperstimulation leads to Leydig cell hyperplasia/hypertrophy in prepubertal mice, which diminishes in adulthood [9, 10].

With the aim to analyze the participation of the TGF- β 1 system in hyperplasia/hypertrophy of Leydig cells, we evaluated the following things in 3-week-old mice of the FVB/n strain: 1) the *in vitro* effect of TGF- β 1 and progesterone on proliferation markers (Proliferating Cell Nuclear Antigen (PCNA) and *Bax/Bcl-2* ratio) and Smad 1/5 activation in purified Leydig cells; and 2) the *in vivo* effect of TGF- β 1 and progesterone on Leydig cell morphometric assessment.

Materials and Methods

Animals

Male mice (3 weeks of age) of the FVB/n strain were bred in our Animal Care Unit (Animal Care Laboratory, Instituto de Biología y Medicina Experimental - Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires). They were kept under controlled temperature (23 C) and lighting conditions (12 h-dark/light cycles). Tap water and commercial mouse chow were provided *ad libitum*. All animal studies were conducted according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (Instituto de Biología y Medicina Experimental - Consejo Nacional de Investigaciones Científicas y Técnicas) following the National Institute of Health (USA) guidelines. Male mice were euthanized by asphyxiation with carbon dioxide (CO₂), and testes were rapidly removed and immediately used for isolation of Leydig

Received: September 25, 2009

Accepted: March 30, 2010

Published online in J-STAGE: April 22, 2010

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cells or fixed for immunohistochemistry.

Preparation of purified mouse Leydig cells

Leydig cells were isolated from testes of mice at 3 weeks of age using a previously described [11] procedure that was adapted and validated to the mouse. The right and left testes of each animal were decapsulated, pooled, immersed in Medium 199 containing 0.1% bovine serum albumin (BSA; Sigma) and 0.2 mg/g tissue collagenase (Sigma Aldrich), at pH 7.2, and incubated for 10 min at 32–34 C with gentle shaking. The incubation was stopped by adding 50 ml of cold Medium 199 without collagenase. The supernatants were filtered (cell strainer; Becton Dickinson and Company, 70 μ m) and centrifuged at 800 g for 10 min. Deoxyribonuclease was added (1.25 mg/ml). The pellet was resuspended and applied to a discontinuous Percoll density gradient and centrifuged (30 min, 1600 g). Cells that migrated to the 1.06–1.12 g/ml density fraction were collected and washed with Medium 199. An aliquot was incubated with 0.4 mg/ml trypan blue and used for cell counting and viability assay using a light microscope. The viability of the Leydig cell preparations (Trypan Blue staining) was 95%. To evaluate enrichment in Leydig cells, the activity of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) was measured as previously described by Levy *et al.* [12]. Cell preparations were 85–90% enriched with mouse Leydig cells.

In vitro incubations of purified Leydig cells

Aliquots (1 ml) of cell suspension in Medium 199 containing 10^6 mouse Leydig cells were incubated in either the presence or absence of TGF- β 1 (1 ng/ml), progesterone (10^{-6} M) or progesterone (10^{-6} M) + TGF- β 1 (1 ng/ml) for 4 h at 37 C under a humid atmosphere of 5% CO₂/95% air. Then, the media were removed, and cells were harvested and kept at –80 C for RNA extraction.

To evaluate PCNA expression 300 μ l of cell suspension in Medium 199 containing 10^5 Leydig cells were incubated with progesterone (10^{-6} M) for 3 h and then stimulated with TGF- β 1 (1 ng/ml) for 1 hour in 8-well chambers (LabTek, Nunc) at 37 C under a humid atmosphere of 5% CO₂/95% air. The media were then removed, and the cells were fixed in paraformaldehyde (4%) and dehydrated with 70–100% ethanol. The cells were used for immunocytochemical studies.

RNA isolation and semi quantitative RT-PCR

Total RNA from purified Leydig cells was extracted with an RNeasy kit (Qiagen), according to the manufacturer's instructions. Total RNA (0.4 μ g) was used for reverse transcription with MMLV reverse transcriptase (Promega). Reverse-transcribed cDNAs (5 μ l) were used to amplify mRNA sequences by a PCR reaction with Go-TaqDNA polymerase (Promega) and the following specific forward (F) and reverse (R) primers: 5' ACACCTGAGCTGACCTTG 3', F, and 5' CGCTCACGGAGGAAGTC 3', R for Bax and 5' CTGCAGAACCTCCCTGTG 3', F, and 5' CGCCGGGGCTTCTTCTTC 3', R, for Bcl-2. The first denaturation was performed for 4 min at 94 C. The successive denaturation steps were performed for 1 min at 94 C followed by annealing at a specific temperature (55 C for both primers) for 1 min and extension at 72 C for 90 sec. Thirty cycles were used for

each pair of primers. A final elongation for 7 min at 72 C was used to amplify transcripts. The housekeeping gene used was β -actin (25 cycles). The amplified cDNA fragments were size-fractionated in 2% agarose gel and visualized by staining with ethidium bromide. The intensity of bands obtained by electrophoresis was evaluated using the Scion Image for Windows software. Results are expressed as the ratio between the expression of the specific gene and β -actin.

Immunocytochemical studies

Cells fixed in an 8-well chamber were washed with ethanol (100–70%) and then washed with PBS. Endogenous peroxidase activity was inhibited with 0.5% v/v H₂O₂ in methanol for 20 min at room temperature. Then, the cells were subjected to saponin (5 mg/ml, 5 min) and proteinase K (20 μ g/ml, 90 sec) treatment for antigenic recovery. The cells were blocked for 1 h with 10% v/v normal goat serum in PBS- 1% BSA and then incubated overnight at 4 C with primary antibody (anti-PCNA, sc-56, Santa Cruz; 1:100).

After five rinses in PBS, the cells were incubated for 1 h at RT with biotinylated secondary antibody (Vector Labs, 1:1000). After further washing in PBS, the cells were incubated for 1 h with streptavidin-peroxidase complex diluted 1:100 (ABC kit, Vector Labs). They were then washed twice with PBS, and development of peroxidase activity was performed with 0.05% w/v 3,3'-diaminobenzidine and 0.1% v/v H₂O₂ in Tris-HCl; they were subsequently subjected to a final wash with distilled water. Negative controls were processed simultaneously by omitting the primary antibodies.

Immunoblotting

Approximately, 2×10^6 mouse Leydig cells were homogenized in 20 mM Tris-HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (IGEPAL CA-630, Sigma) and 1% of a preformed mixture of protease inhibitors (Sigma). Samples were heated at 95 C for 5 min under reducing conditions (10% mercaptoethanol). Homogenates (220 μ g protein) were loaded onto tricine-SDS-polyacrylamide gels (10%), electrophoretically separated and blotted onto nitrocellulose. Protein concentrations were measured by the method of Lowry *et al.* [13]. Blots were incubated with rabbit polyclonal anti-Smad 1/5/8 (N-18)-R (sc-6031-R, Santa Cruz Biotechnology, 1:200) or rabbit polyclonal anti-p-Smad 1/5/8 (#9511, Cell Signaling Technology, Beverly, MA, USA; 1:200), and subsequently with the peroxidase-labeled secondary antibody goat anti-rabbit IgG (1:5000, Sigma). Signals were detected with an ECL kit (Amersham Pharmacia Biotech AB). The intensity of bands obtained by electrophoresis was evaluated using the Scion Image for Windows software.

Testis morphometry

To study testicular morphometry, mice were injected with progesterone (s.c. 20 mg/Kg) or castor oil at 3 weeks of age. After 24 h, the animals received an intratesticular injection of TGF- β 1 (1 μ l, 1.5 ng/100 μ l). Control animals were injected with 1 μ l of saline. The animals were euthanized 24 h later.

Testes were immersed in 4% buffered glutaraldehyde for 48 h

and then washed with 0.5 M phosphate buffer. Tissues were embedded in glycol methacrylate, and subsequently, sections 4 μm in thick were obtained and stained with toluidine blue.

The volume densities of the testicular tissue components were determined by light microscopy using a 441-intersection grid placed in the 10 $\times$ ocular of a light microscope [14]. Fifteen fields chosen randomly (6615 points) were scored for each animal at 400 $\times$ magnification. Artifacts were rarely seen and were not considered in the total number of points utilized to obtain volume densities. Points were classified as one of the following: seminiferous tubule, comprising tunica propria, epithelium and lumen; Leydig cells; blood vessels and lymphatic spaces; or connective tissue. The volume of the each component of the testis was determined as the product of the volume density and testis volume.

The individual volume of Leydig cells was obtained from their nuclear volume and the proportion of the nucleus and cytoplasm. To calculate the proportion of the nucleus and cytoplasm, a 441-point square lattice was placed over the sectioned material at 1000 $\times$ magnification. One thousand points over the Leydig cells were counted for each animal. The Leydig cell nuclear volume was expressed in μm^3 and obtained by the formula $4/3\pi r^3$, where r =nuclear diameter/2. The number of Leydig cells per testis was estimated from the Leydig cell individual volume and the volume occupied by Leydig cells in the testis parenchyma [14].

Statistical analysis

Mean and standard error (SEM) were calculated. GraphPad Prism (version 3.00 for Windows, GraphPad software, San Diego, CA, USA) was used for one-way analysis of variance. Tukey's *t*-test and the Student's *t*-test were used when differences between two or more than two groups were compared, respectively. A *P*-value of less than 0.05 was considered significant.

Results

Effect of TGF- β 1 and progesterone on proliferation markers in Leydig cells

Incubation of purified Leydig cells from 3-week-old mice with progesterone (10^{-6} M) did not induce PCNA expression (Fig 1, panel B). However, the incubation with TGF- β 1 (1 ng/ml) showed a slight increase in the induction of this proliferation marker (Fig 1, panel C). Moreover, the incubation with TGF- β 1 (1 ng/ml) plus progesterone (10^{-6} M) clearly increased PCNA expression with respect to the control (Fig 1, panels D and A, respectively).

Furthermore, purified Leydig cells were incubated with TGF- β 1 (1 ng/ml) and progesterone (10^{-6} M), and the gene expression of *Bax* and *Bcl-2* was analyzed by RT-PCR. Fig. 1 (panel E) shows that progesterone in the presence or absence of TGF- β 1 decreased the ratio of *Bax/Bcl-2*, which reflects the apoptotic index, provided that *Bax* gene expression diminished without changes in *Bcl-2* gene expression.

Effect of TGF- β 1 and progesterone on p-Smad 1/5

In order to study the molecular events involved in the TGF- β 1 transduction pathway, we investigated the protein expression of Smad 1/5, which is activated by phosphorylation and is involved in

Fig. 1. Effect of progesterone and TGF- β 1 on PCNA expression. Purified Leydig cells from mice at 3 weeks of age were incubated under basal conditions (panel A) or stimulated with progesterone (10^{-6} M; panel B), TGF- β 1 (1 ng/ml; panel C) or progesterone + TGF- β 1 (panel D). PCNA was revealed by immunocytochemical detection. Effect of progesterone and TGF- β 1 on *Bax* and *Bcl-2* gene expression (panel E). Purified Leydig cells from mice at 3 weeks of age were incubated under basal conditions or stimulated with progesterone (10^{-6} M), TGF- β 1 (1 ng/ml) or progesterone + TGF- β 1. *Bax* and *Bcl-2* were evaluated by semiquantitative PCR reaction. Results are represented with an arbitrary scale and expressed as the ratio between the expressions of *Bax*, *Bcl-2* and β -actin (integrated density, ID). Data are plotted as the mean ID $\pm$ SEM (n=5) of one experiment out of three. Effect of progesterone and TGF- β 1 on p-Smad 1/5/8 expression (panel F). Purified Leydig cells from mice at 3 weeks of age were incubated under basal conditions or stimulated with progesterone (10^{-6} M) + TGF- β 1 (1 ng/ml). Smad 1/5/8 and p-Smad 1/5/8 were evaluated by Western blot. Results for the lower band are represented with an arbitrary scale and expressed as the ratio between the expression of p-Smad 1/5/8 and Smad 1/5/8 (integrated density, ID). Data are plotted as the mean ID $\pm$ SEM (n=5) of one experiment out of three.

proliferation events triggered by this cytokine. TGF- β 1 (1 ng/ml) in the presence of progesterone (10^{-6} M) increased the expression of p-Smad 1/5 (Fig. 1, panel F). A doublet of bands was observed in the TGF- β 1 plus progesterone incubates, with the upper band

Fig. 2. Volume densities of the testicular tissue (panels A and B) and morphometric data of Leydig cells (panels C, D and E) of mice at 3 weeks of age. Animals were injected with progesterone (s.c. 20 mg/Kg) or castor oil. After 24 h, the animals received an intratesticular injection of TGF- β 1 (1 μ l, 1.5 ng/100 μ l). The mice were euthanized 24 h later. Data are plotted as the mean $\pm$ SEM (n=5).

only present in these incubates and the lower band observed also under the basal conditions but with lower intensity.

Testis morphometry

The volume of seminiferous tubule (%) decreased and that of the interstitium (%) increased in animals treated with progesterone plus TGF- β 1 and progesterone with respect to the control group (Fig 2; panels A and B, respectively). In spite of showing a slight increase in Leydig cell number in the TGF- β 1 and progesterone experimental group, there were no significant differences between groups (data not shown). However, in the mice treated with TGF- β 1 plus progesterone, the nuclear volume decreased and the cytoplasmic volume increased in Leydig cells respect to the control group (Fig. 2, panels C and D, respectively). Thus, Leydig cell volume was significantly increased in this experimental group (Fig. 2, panel E).

Discussion

One of the most important functions of TGF- β 1 is regulation of cell cycle progression. Different observations have been made about this effect. It has been described that TGF- β 1 stimulates apoptosis of endothelial, epithelial, fibroblastic and hematopoietic cells, but at the same time, is capable of promoting proliferation in epithelial and endothelial cells [15]. This dual effect on cell proliferation has been extensively described in endothelial cells, where high doses of TGF- β 1 inhibit cell cycle progression and low doses

of this cytokine exert the opposite effect [7, 16].

It might be possible that increased levels of endogenous TGF- β 1 caused by some pathological conditions besides proliferation of Leydig cells trigger the Leydig cell hyperplasia / hypertrophy. Thus, the present study would provide the basic molecular information for understanding this particular testicular pathogenesis.

Immunocytochemical studies on purified Leydig cells incubated with low concentrations of TGF- β 1 plus progesterone showed a clear increase in PCNA expression, while TGF- β 1 alone had a slight effect on this proliferation marker. Progesterone alone did not modify PCNA immunodetection; however, it has been observed that progesterone stimulates endoglin gene expression in mouse Leydig cells [17]. Moreover, incubation of Leydig cells with TGF- β 1 and progesterone diminished the expression of *Bax* (pro-apoptotic gene) without any modification of *Bcl-2* (anti apoptotic gene) expression, causing a lower *Bax/Bcl-2* ratio compared with the control treatments.

Furthermore, we observed a clear increase in Smad1/5 phosphorylation in Leydig cells treated with TGF- β 1 plus progesterone. A doublet of bands was observed in the western blot detection of phospho-Smad 1/5/8. In this context, some authors have also observed multiple bands in western blot detection of this protein [18, 19]. Daly *et al.* [18] identified these bands by a siRNA knock-down of all the individual Smad proteins and established that the upper band is comprised mainly of Smad1, with some Smad5, whereas the lower band is an isoform of Smad5.

The results of the present study are in agreement with those of Lebrin *et al.* [20] and Goumans *et al.* [21]. These authors pointed out that a low dose of TGF- β 1, via the ALK1/Smad1/5 signaling pathway, leads to epithelial cell proliferation. In this context, the coreceptor endoglin plays a key role. It has been observed that endoglin is required to induce efficient ALK1 signaling, which inhibits the ALK5 signaling pathway [7, 20]. These observations suggest that, in prepubertal Leydig cells, TGF- β 1 acting in concert with progesterone could stimulate proliferation.

Since purified Leydig cells do not proliferate *in vitro*, we evaluated the *in vivo* effect of TGF- β 1 and progesterone on Leydig cell hyperplasia/hypertrophy. Morphometric studies revealed that animals treated with TGF- β 1 plus progesterone or progesterone alone presented an increment in the volume of the interstitial tissue with a decrease in the tubular compartment volume. Moreover, we observed that in mice treated with TGF- β 1 and progesterone, Leydig cells showed a decrease in nuclear volume with a concomitant increase in the cytoplasmic volume with respect to the other groups. Together, these results suggest the involvement of TGF- β 1 and progesterone in Leydig cell hypertrophy. To our knowledge, there are no reports in the literature that have analyzed the *in vivo* effect of TGF- β 1 on hypertrophy of Leydig cells. In the present work, the treatment with TGF- β 1 plus progesterone failed to modify significantly the number of Leydig cells, in spite of a slight increase in this experimental group with respect to the control mice. This result might suggest that prolonged *in vivo* treatments are required to produce a hyperplastic effect.

In summary, based on the parameters determined in this study, we are prompted to suggest that low doses of TGF- β 1 in concert with progesterone, are involved in the process of hypertrophy/

hyperplasia of Leydig cells. We expect that these results will shed some light on the physiological role of TGF- β 1 in male reproductive health, since a number of testicular pathologies (Sertoli Cell-Only Syndrome, Hypospermatogenesis) in infertile men are associated with hyperplasia and hypertrophy of Leydig cells [22].

Aknowledgment

This study was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Técnica (ANPCyT) and Facultad de Medicina-Universidad de Buenos Aires.

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