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Expression and Regulation of Transforming Growth Factor β1 mRNA and Protein in Rat Fetal Testis *in Vitro*

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The expression and secretion of Transforming Growth Factor β1 (TGFβ1) by cultured testes of day 20.5 rat fetuses were investigated. The testes were found to express two TGFβ1 mRNA transcripts of 2.5 and 1.8 kb. By using mink lung epithelial cell bioassay based on the measurement of the inhibition of tritiated thymidine incorporation in response to TGFβ1 immunoreactive material, the fetal testes were shown to secrete TGFβ1 protein in organ culture. This secretion was positively regulated by dibutyryl cyclic AMP or by LH and FSH together, but not by LH alone and very slightly by FSH alone, which suggests interactions between Leydig and Sertoli cells for the control of TGFβ1 production. These regulations probably take place at a posttranscriptional step since no concomitant increase of TGFβ1 mRNA levels was observed. Such a positive regulation of TGFβ1 secretion by gonadotropins could be a characteristic of the rat fetal testis.

The Transforming Growth Factor βs (TGFβs) are secreted growth and differentiation factors that exert a variety of biological activities depending on the target cell type (1-3). Three different isoforms named TGFβ1, TGFβ2, TGFβ3 have been identified in mammals (4). The activity of secreted TGFβ is masked by its propeptide (LAP) which is cleaved from the mature dimeric TGFβ in the secretory pathway (5), but remains associated with the mature dimer by non covalent interactions (6). In vitro, TGFβ can be activated by acid treatment or proteolysis. Biochemical and genetic evidence has shown that TGFβ family members signal by simultaneously contacting two transmembrane serine/threonine kinases known as the type I and type II receptors. Both receptors are required for TGFβ action in mammalian cells (7, 8). Following interaction with its cell surface receptors, TGFβ has the ability to inhibit (epithelial cells) or stimulate (mesenchymal cells) proliferative activity. TGFβ also affects cell differentiation (3). Especially, TGFβ1 has been implicated as a paracrine/autocrine regulator of testicular functions since the testis is a site of expression and production of TGFβ1 (9-15). Moreover, homozygous mutant TGFβ1-deficient mice exhibit abnormalities in reproductive function (16) and TGFβ1 has been shown to be a potent inhibitor of differentiated functions of pig (17, 18), rat (19) and mouse (20) Leydig cells and of porcine Sertoli cells (21).

In a recent immunohistological study we have shown that TGFβ1 is present very early in the rat fetal testis (22) and that this factor is able to inhibit steroidogenesis by fetal Leydig cells in culture (23) and to reduce the number of gonocytes in the fetal testis in vitro (24). In the present study, we investigated the expression and secretion of TGFβ1 by the fetal testis in organ culture and their regulation by gonadotropins.

MATERIALS AND METHODS

Chemicals/solutions. The culture medium was Medium 199 containing 4.18 mM sodium bicarconate, 0.35% glutamine and 5 μg/ml transferrin (Sigma) and for TGFβ1 assay was Ham's F12/Dulbecco's Modified Eagle's Medium (1:1). The media as well as trypsin-EDTA solution were purchased from Gibco (Grand Island, NY). Gentamicin (Gentalline, Scherring-Plough, Levallois-Perret, France) was added at 40 μg/ml. Ovine (o)LH (NIH LH S19; 1.01 NIH LH S1 units/mg) was a gift from the NIH (NIDDK, Bethseda, MD). Recombinant hFSH (12000 IU/mg) was a gift from Dr. B. Mannaerts (Organon International, Oss, The Netherlands). Porcine TGFβ1 was purchased from R&D Systems (Minneapolis, MN). 4β-phorbol 12 myristate 13-acetate (PMA), bovine serum albumin (BSA), dibutyryl cyclic AMP (dbcAMP), 3-isobutyl-1-methyl-xanthine (IBMX) were purchased from Sigma Chemical Co (St Louis, MO).

Antibody. The anti-TGFβ1 polyclonal antibody was raised by immunizing rabbits with a synthetic peptide corresponding to amino acids 91-103 of mature TGFβ1. This antibody has been extensively characterized (14, 22). It is specific for TGFβ1 and does not cross react with TGFβ2 or TGFβ3.

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Hybridization probes. A 1.05 kb EcoRI fragment of human TGFβ1 cDNA (gift from R. Derynck, Genetech, Inc., San Francisco, CA) was cut by PvuII in a 0.243 kb fragment and labeled to a specific activity of 10^9 dpm/μg DNA by using [α-^{32}P] dCTP and Amersham's Megaprime DNA labeling kit (Amersham France, Les Ulis, France).

Animals. Female rats of the Wistar strain from Iffa Credo (l'Arbresles, France) were housed under a controlled photoperiod (lights on from 06 00 h - 20 00 h) and fed on a commercial diet (UAR, Villemoisson sur Orge, France) with tap water ad libitum. Males were caged with females for the night and the day following an overnight mating was counted as day 0.5. On day 20.5 of gestation, pregnant rats were anesthetized by an intraperitoneal injection of 4 μg/100 g body weight of sodium pentobarbital (Sanofi, Libourne, France) and the fetal testes were removed aseptically.

Organ cultures. Cultures were performed on Millipore culture filters (Bedford, MA) as previously described (25, 26). Briefly each testis were cut into eight pieces and the pieces from 8 to 10 testes placed on a Millipore filter (pore size : 0.45 μm). The filter, with the pieces of testes on its top surface, was floated on 1.5 ml of culture medium (Medium 199) in tissue culture dishes 35 mm in diameter. The testes from one side of the fetuses were cultured in control medium and the pieces from the contralateral testes in medium containing the hormones or factors as indicated. Cultured were continued for 72 h at 37°C, in a humidified atmosphere of 5% O_2 in air. Media were collected and changed every 24 h.

TGFβ1 biossay. Conditionned media from two dishes were pooled, acidified with glacial acetic acid to a final concentration of 1 M, concentrated 20/30 times by ultrafiltration using Microsep microconcentrators (Filtron, Northborough, MA), lyophilizated and frozen at −20°C until assay. For TGFβ1 bioassay, each sample was resuspended in assay medium, neutralized and tested using mink lung epithelial (CCL-64) cells. These cells were obtained from the American Type Culture Collection (Rockville, MD; N CRL 6534) and maintained in assay medium supplemented with 10% fetal calf serum. TGFβ1 induced DNA synthesis inhibition in CCL-64 cells was carried out as described by Danielpour et al. (27) with minor modifications from Avallet et al. (14). In 0.32 cm^2 wells (96 wells plate; Falcon, Grenoble, France), aliquots (10-20 μl) of the samples were added in 100 μl assay medium supplemented with 5% fetal calf serum. CCL-64 cells in logarithmic growth phase were trypsinised, washed once with assay medium and plated at 3×10^4 cells/100 μl in the wells. CCL-64 cells were cultured overnight (16-20 h), followed by pulse labeling with [^{3}H]thymidine (28 Ci/mmol; Dositek, Orsay, France) at a final concentration of 1 μCi/ml for 4 h at 37°C. The cells were fixed with 80% methanol-acetic acid (3:1, vol/vol) and were washed twice with 80% methanol. ^{3}H-labeled DNA was extracted by 250 μl 0.4% deoxycholate 0.5 N NaOH. Radioactivity was measured by a liquid scintillation counter. Standard bioassay curve was established with the same procedure. Half-maximal and maximal inhibition were observed at 50 and 300 pg TGFβ1/ml respectively. At 1:20 dilution, the antibody completely blocked the inhibitory effect of 200 pg/ml TGFβ1 on [^{3}H]thymidine incorporation in CCL-64 cells (controls 100%; TGFβ1 9.6 ± 2.1%; TGFβ1 plus antibody 78.2 ± 2.2%, n = 6). When the samples were tested with 10 and 20 μl, dose-dependent inhibition was obtained. The sample induced DNA synthesis inhibition in CCL-64 was blocked by the antibody (1:20 dilution), [^{3}H]thymidine incorporation being 90 ± 4% (n = 9) of control values.

Northern blot analysis. At the end of the culture, the testes were harvested in guanidine thiocyanate. Total RNA (20 or 30 μg) was extracted (28), denatured by heating (65°C for 15 min) and subjected to electrophoresis through a 1% agarose gel containing 10% formaldehyde and transferred to Hybond-N nylon membrane (Amersham). Hybridization was performed according to Avallet et al. (14). Autoradiographs were obtained after 4-8 days exposure at −70°C to Kodak X-OMAT-AR films (Eastman Kodak, Rochester, NY). For all Northerns, photos were taken of ethidium bromide-stained blots using Polaroid 665 film. Signal intensity of the autoradiograms and of the 28S band on the negative were quantified with a computerized video densitometer (Biocom, les Ulis, France). The data were expressed as the absorbance of the mRNA band/absorbance of the 28S band.

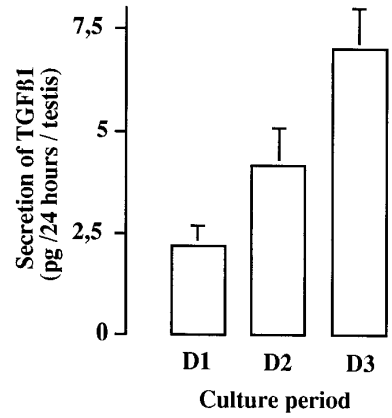

FIG. 1. Secretion of TGFβ1 by fetal testes explanted on day 20.5 and cultured for 3 days (D1, D2, D3) on Millipore filters in defined medium. Conditioned media were collected every 24 hours, acidified, concentrated, and tested for TGFβ1 concentration using CCL-64 epithelial cell bioassay. Results are means ± SEM of 14 separate experiments.

Immunohistochemistry. In some experiments, at the end of the culture, testes pieces were fixed in Carnoy's liquid (ethanol-chloroform-acetic acid, 6/3/1) for 2 h at 20°C. Paraffin sections were used for TGFβ1 immunostaining as previously described (22, 29).

RESULTS

Secretion of TGFβ1 by fetal testes in vitro. [^{3}H] thymidine incorporation into CCL-64 cells was tested in the presence of acidified conditionned media from fetal testes. The results show that control testes do secrete TGFβ1 and that this secretion increases with time (Figure 1), reaching about 7 pg/24 h/testis on the third day of culture. Secretion of TGFβ1 was measured in the presence of gonadotropins or dbcAMP from the beginning of the culture (Figure 2). LH alone (100 ng/ml) did not modified TGFβ1 secretion while FSH alone (200 mU/ml) or in the presence of IBMX (1 mM) increased slightly but significantly this secretion. The presence of LH plus FSH or dbcAMP (1 mM) stimulated dramatically TGFβ1 secretion which reached 300% of control values on the third day of culture. This increase was already detectable on the second day of culture (172 ± 55% of control values for dbcAMP compared to controls, n = 4, $p < 0.05$). The addition of PMA (10^{-7} M) did not alter TGFβ1 secretion.

Expression of TGFβ1 mRNA. Northern blot analysis of total mRNA extracted from fetal testes after 3 days culture are shown in Figure 3. After hybridization with a radiolabeled human TGFβ1 cDNA, two mRNA species were detected, a major 2.4 kb band specific of TGFβ1 and a weaker 1.8 kb band. Identical transcripts were observed after hybridization of mRNA extracted

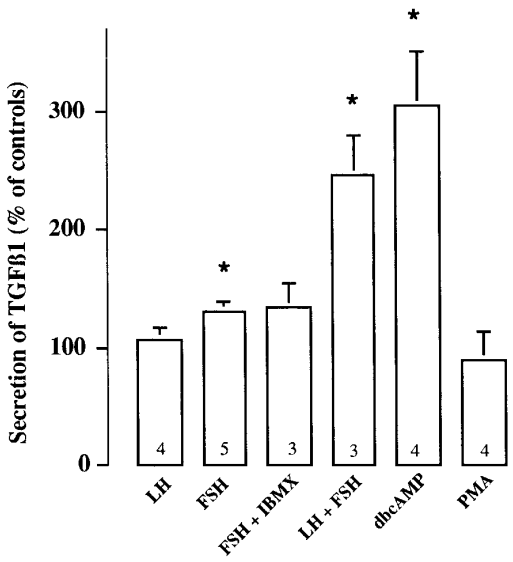

FIG. 2. Secretion of TGFβ1 by fetal testes explanted on day 20.5 and cultured for 3 days on Millipore filters in control medium (100%) or in the presence of 100 ng/ml LH, 200 mU/ml FSH, FSH plus IBMX (1 mM), LH plus FSH, 1 mM dbcAMP, or 10^{-7}M PMA. Media were changed every 24 hours. On the third day, the conditioned media were acidified, concentrated, and tested for TGFβ1 concentration using CCL-64 epithelial cell bioassay. Results are expressed as a percentage of control values (means ± SEM) on the third day of culture. n (number of separate experiments) is indicated at the base of the columns. *, $p < 0.05$.

from freshly isolated testes of 20.5 day fetuses (result not shown). Quantitative analysis of the Northern blot from testes cultured for 3 days in the presence of FSH, LH, or LH plus FSH (Figure 3), revealed no significant difference of TGFβ1 mRNA levels with control values. The testes cultured in the presence of dbcAMP exhib-

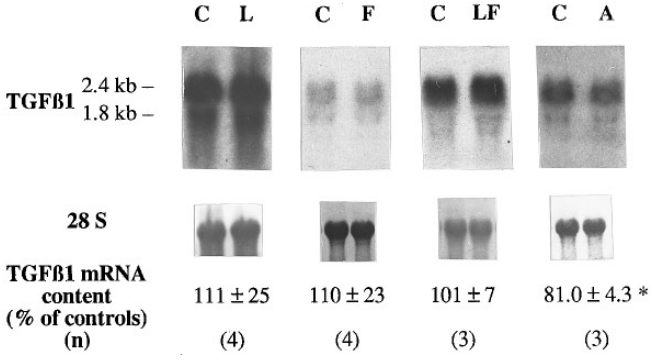

FIG. 3. Northern blot analysis of TGFβ1 mRNA levels in fetal testes explanted on day 20.5 and cultured for 3 days on Millipore filters in control medium (C) or in the presence of 100 ng/ml LH (L), 200 mU/ml FSH (F), LH plus FSH (LF), or 1 mM dbcAMP (A). Autoradiographs were quantified by computerized video densitometry and normalized to 28S signal. Results are expressed as a percentage of control values (means ± SEM). (n) number of separate experiments. *, $p < 0.05$.

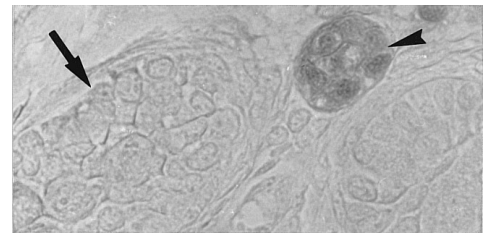

FIG. 4. Immunohistochemical localization of TGFβ1 in fetal testis explanted on day 20.5 and cultured for 3 days on Millipore filters in defined medium. Leydig cells (arrowhead) exhibited intense staining for TGFβ1 while no staining was detected in the seminiferous cords (arrow).

ited a slow but significant decrease of TGFβ1 mRNA level.

Immunohistochemistry. In some experiments, immunostaining of TGFβ1 was performed on testis pieces after 3 days of culture. Intense staining for TGFβ1 was observed only in the Leydig cells (Figure 4), that is to say in the same localization as previously observed in freshly isolated testes (22). The testes cultured in the presence of LH, FSH, LH plus FSH or dbcAMP revealed similar staining (results not shown).

DISCUSSION

In the present study we have shown that extracts from testes of day 20.5 rat fetuses contain two TGFβ1 mRNA species of 2.4 and 1.8 kb. These two species were observed before and after 3 days of culture. The major species of 2.4 kb has been classically reported in various tissues and in somatic testicular cells of pig (14), rat (10). In the mouse, both transcripts have been described and, in the testis, the 1.8 kb appears to originate from germ cells (12).

TGFβ1 bioassay demonstrated that the rat fetal testis secrete TGFβ1-like activity which was indeed due to authentic TGFβ1 since TGFβ1 antibody neutralize 90% of this bioactivity. The TGFβ1 antibody used in the study has been extensively proved to recognize with high affinity TGFβ1 but not the two other isoforms found in mammals, TGFβ2 or TGFβ3 (14, 22). In immunolocalization studies, we have previously revealed that TGFβ1 (22), TGFβ2 (30) and TGFβ3 (unpublished observations) are present in fetal rat Leydig cells on fetal day 20.5. Although not quantitative, these studies have shown an immunostaining of particularly high intensity for TGFβ1 isoform which is in agreement with the present observation that TGFβ1 is secreted in particularly large amounts, TGFβ2 and TGFβ3 being probably secreted in neglectable quantities. Other studies had reported that Sertoli and peritubular cells from 20 day-old rats secrete the three isoforms of TGFβ (10, 11) but that pig Sertoli and Leydig cells secrete TGFβ1 in culture (14). If the fetal testis secrete TGFβ1

in a latent or in a bioactive form is presently unknown. In the present study the bioactivity of the conditioned media from the fetal testes were tested only after activation by acidification because these media were not available in large amounts. However, in cultures of bovine luteal cells (31) or of pig Sertoli and Leydig cells (14), TGFβ1 has been shown to be secreted in a latent form.

Concerning the regulation of TGFβ1 secretion, we did not observe any effect of LH on the fetal testis, while FSH induced a weak stimulatory effect which was not amplified by IBMX. However, when added together, the gonadotropins induced a strong increase of TGFβ1 secretion. A similar stimulation was reproduced by dbcAMP. Therefore, the present results strongly suggest the existence of relationships between Sertoli and Leydig cells for TGFβ1 secretion. Taking into account of the immunohistochemical study, we can suppose that, into the testes of day 20.5 fetuses, the cells responsible for TGFβ1 secretion are the Leydig cells. One hypothesis could be that FSH induces the production by Sertoli cells of factor(s) able to stimulate the production of TGFβ1 by the Leydig cell and this stimulation would be of great amplitude only if the Leydig cells were stimulated by LH or dbcAMP. The quantification of TGFβ1 mRNA in the fetal testes after 3 days culture in the presence of gonadotropins or dbcAMP did not reveal any increase of the mRNA levels above control values. This points out a discrepancy between the secretion of the protein and the expression of its mRNA. The regulation of TGFβ1 secretion by the gonadotropins and dbcAMP results therefore probably from a posttranscriptional regulation. Indeed, a slight decrease of the TGFβ1 mRNA level was observed in the testes cultured in the presence of dbcAMP which could be due to a degradation of the mRNA as it is highly translated (32). In several cell types such a discrepancy between TGFβ1 mRNA and protein levels has been reported and was indebted to a posttranscriptional regulation (33). For example, in cells from prostatic carcinoma which secrete predominantly TGFβ2 but relatively little TGFβ1 protein, the TGFβ1 mRNA level is higher than that of TGFβ2 (34, 35). In primary mouse keratinocytes, retinoic acid increases the steady state levels of TGFβ1 mRNA but there is no corresponding increase in secretion of TGFβ1 protein (36). Furthermore, activation of monocytes with lipopolysaccharides induces TGFβ1 secretion with no change in mRNA levels (37). It has been recently shown that the 5′-untranslated region (UTR) of the TGFβ1 mRNA plays an important role in the posttranscriptional regulation of TGFβ1 expression and particularly in the inhibition of TGFβ1 translation (33).

Concerning the regulation of TGFβ secretion by the testis, FSH has been reported to inhibit the secretion of a TGFβ-like protein by immature pig Sertoli cells (38) and to reduce the TGFβ1 mRNA level (14). In Sertoli cells from 20 day-old rats, FSH does not alter the secretion of TGFβ1 nor TGFβ3 but reduces the TGFβ2 mRNA level and the secretion of the TGFβ2 protein (11). The single report concerning Leydig cells has been performed with immature pig Leydig cells in culture and has shown that LH does not modify the TGFβ1 mRNA level (14).

In conclusion, in the testis, it seems that there is a great diversity in the regulation of the TGFβs according to the isoforms, the species and/or the developmental stage. However a positive regulation of gonadotropins on the TGFβ1 secretion could be a characteristic of the rat fetal testis. During late fetal life, since gonadotropins secretion increases (39) and since TGFβ1 has been shown to be a potent local inhibitor of fetal Leydig cell steroidogenesis (23), this factor is postulated to be implicated in the decrease of testosterone secretion that occurs during this period.

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