Effects of transforming growth factors- α and - β on proliferation and apoptosis of rat theca-interstitial cells

T Pehlivan, A Mansour, R Z Spaczynski¹ and A J Duleba

Yale University School of Medicine, Department of Obstetrics and Gynecology, 333 Cedar Street, New Haven, Connecticut 06520-8063, USA

¹Karol Marcinkowski University School of Medical Sciences, Department of Gynecology and Obstetrics, ul. Polna 33, Poznan, Poland

(Requests for offprints should be addressed to A J Duleba)

Abstract

Ovarian development, follicular growth and atresia require mechanisms regulating proliferation and death of ovarian cells including theca-interstitial (T-I) cells. Transforming growth factors- α and - β (TGF- α and TGF- β) are well recognized local modulators of T-I function. This study was performed to evaluate the effects of TGF- α and TGF- β on ovarian T-I cell proliferation, differentiation and apoptosis. T-I cells from immature Sprague-Dawley rats were purified and incubated in chemically defined media. Proliferation was assessed by [³H]thymidine incorporation assay and by cell counting. Steroidogenically active cells were identified histochemically by detection of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity. DNA was extracted and apoptosis was identified by detection of internucleosomal DNA cleavage producing the characteristic 'ladder pattern' of low-molecular weight (LMW) DNA following agarose gel electrophoresis. Quantification of apoptosis was carried out with the aid of 3'-end labeling of DNA fragments with [³²P]-dideoxy-ATP. TGF- α and TGF- β stimulated [³H]thymidine incorporation by 2.2- to 3.1-fold and 1.7- to 3.4-fold respectively ($P<0.005$). A combination of TGF- α and TGF- β produced a synergistic increase in DNA synthesis by 6.7-fold (at 1 ng/ml of each TGF- α and TGF- β ;

$P<0.001$) and tenfold (at 10 ng/ml of each TGF- α and TGF- β ; $P<0.001$). Cell counting revealed that TGF- α increased the total number of cells 2.8-fold and TGF- β 2.8-fold. The combination of TGF- α and TGF- β increased the total cell count 3.2-fold, compared with control ($P<0.05$). The percentage of the steroidogenically active cells was $37\pm9\%$ (mean $\pm$ s.e.m.) in the control cultures, $50\pm5\%$ in the presence of TGF- α , $42\pm8\%$ in the presence of TGF- β , and $47\pm13\%$ in the presence of both TGF- α and TGF- β . TGF- α decreased apoptosis by $63\pm14\%$ ($P=0.02$) while TGF- β had no statistically significant effect. TGF- α in combination with TGF- β produced the greatest inhibition of apoptosis by $73\pm8\%$ ($P=0.01$). These findings demonstrate that TGF- α and - β stimulate proliferation of both steroidogenically active and inactive T-I cells. Furthermore, TGF- α alone and in combination with TGF- β protects T-I cells from apoptotic death. These effects of TGFs may be important in physiologic maintenance of ovarian mesenchymal growth and homeostasis as well as in pathophysiologic conditions associated with excessive growth of the T-I compartment, such as polycystic ovary syndrome.

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Introduction

Ovarian growth during development and subsequent maintenance of ovarian function, including folliculogenesis, and atresia require precise mechanisms regulating growth and activity of the granulosa and thecal/stromal compartments. The processes of growth or regression of tissues, i.e. an increase or a decrease in the number of cells, are controlled by modulating the rate of proliferation and/or death of cells. These processes are under the control of an intricate network of endocrine, paracrine and autocrine interactions. Proliferation and apoptosis of ovarian cells are regulated by gonadotropins, ovarian steroids and various growth factors (Harman *et al.* 1975, Hurwitz & Adashi 1992, Tilly *et al.* 1992, Hsueh *et al.* 1994).

Transforming growth factors- α and - β (TGF- α and TGF- β) are well known locally produced modulators of ovarian function. TGF- α is single-chain, 50-amino acid polypeptide with structural similarity to epidermal growth factor (EGF). Both TGF- α and EGF bind to the same receptor initiating growth and proliferation in a broad range of cell types (Massague 1983, Tam *et al.* 1984). Their receptors have been detected in ovarian cells of many species, including chicken, rat and human (Onagbesan *et al.* 1994a, Scurry *et al.* 1994, McWilliam *et al.* 1995, Tekpetey *et al.* 1995, Onagbesan & Peddie 1998). Expression of TGF- α gene and production of TGF- α has been demonstrated in granulosa and theca cells from various species (Kudlow *et al.* 1987, Skinner & Coffey 1988, Onagbesan *et al.* 1994a).

TGF- β is a member of a superfamily of peptides which also includes inhibin, activin, and mullerian inhibiting substance. Three mammalian TGF- β genes encode for TGF- β 1, TGF- β 2, and TGF- β 3; these peptides appear to induce similar effects but with different potencies depending on the cell type and assay used (Graycar *et al.* 1989). TGF- β acts in an autocrine and paracrine fashion; among its many functions, it plays an important role in the regulation of growth, development and tissue repair (Hernandez *et al.* 1990, Clark & Coker 1998). Immunolocalization, secretion, and expression of TGF- β in theca and granulosa cells has been shown in several species including rat and human (Gangrade & May 1990, Hernandez *et al.* 1990, Mulheron *et al.* 1991, Chegini & Flanders 1992, Chegini & Williams 1992, Foghi *et al.* 1997). Receptors to TGF- β have been detected in granulosa, and theca cells of various species including rodent and human (Schmid *et al.* 1994, Juneja *et al.* 1996, Roy & Kole 1998, Qu *et al.* 2000).

Several lines of evidence indicate that both TGF- α and TGF- β may be important in the regulation of the function and growth of thecal compartment. Both these peptides have been shown to modulate thecal steroidogenesis and follicular differentiation (Hernandez *et al.* 1990, McAllister *et al.* 1994, Fournet *et al.* 1996). At present, the role of TGF- α and TGF- β in the regulation of thecal growth is uncertain and the results of *in vitro* and *in vivo* studies are contradictory. Under various experimental conditions, TGF- α either stimulated thecal proliferation or had no effect on this process (Foghi *et al.* 1997, Skinner & Coffey 1988, Campbell 1999). TGF- β , on the other hand, suppressed thymidine incorporation, but had no effect on the cell number (Roberts & Skinner 1991). Recent reports have indicated that a combination of TGF- α and TGF- β inhibited thecal growth by inducing apoptotic death (Foghi *et al.* 1997, 1998).

This study investigated the effects of TGFs on proliferation, differentiation and apoptosis of theca-interstitial (T-I) cells using a rodent model. The present findings contrast with previous reports and indicate that both TGF- α and TGF- β promote growth of T-I cells.

Materials and Methods

Materials

The following materials were purchased from Sigma Chemical Co. (St Louis, MO, USA): Medium-199 with HBSS, medium 199 with HBSS ($\times 10$), McCoy's 5a medium (modified, without serum), L-glutamine, BSA, trypsin-EDTA (0.05%/0.02%), nitro-blue tetrazolium, 5 β -androstane-3 β -ol-17-one, β -NAD⁺, sesame oil, Percoll, Hoechst 33342 and DiOC5. Collagenase type I (*Clostridium histolyticum*, CLS1; 146 U/mg) and DNase I (bovine pancreas; 2298 U/mg) were obtained from Worthington Biochemical Co. (Freehold, NY, USA). The

following materials were purchased from Grand Island Biological Co. (Grand Island, NY, USA): Trypan blue stain (0.4%; wt/vol.), antibiotic-antimycotic preparation (penicillin, 10 000 IU/ml; streptomycin, 10 000 μ g/ml; amphotericin B, 25 μ g/ml), and Dulbecco's phosphate-buffered saline (PBS, $\times 1$, pH 7.2, without MgCl₂ and CaCl₂). HEPES was purchased from American Bioanalytical (Natick, MA, USA). Radiolabeled [³H]-thymidine, was purchased from Amersham Life Science Inc. (Arlington Heights, IL, USA). Human TGF- α and TGF- β ₁ were purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Immature (25 days old) female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY, USA) and housed with a ratio of 12 h light:12 h darkness in an air-conditioned environment. Standard rat chow and water were given and animals were allowed to feed *ad libitum*. Starting on the 28th day of age the animals were injected with 17 β -estradiol (1 mg/0.3 ml sesame oil s.c.) daily for 3 days in order to stimulate ovarian development. On the morning following the last injection (day 31 of age) the animals were anesthetized with ketamine and xylazine (i.p.) and sacrificed by perfusion with 0.9% saline. All treatments and procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and a protocol approved by the Yale University Animal Care Committee.

Isolation of T-I cells and cell cultures

Ovaries were dissected, and T-I cells were isolated and purified as described previously (Duleba *et al.* 1997). The cells were counted using a hemocytometer and viability was determined using trypan blue stain exclusion test. Cell viability was in the 85–95% range. The cells were cultured in McCoy's 5a medium supplemented with L-glutamine (2 mM), BSA (1 mg/ml), penicillin (10 000 IU/ml), streptomycin (10 000 μ g/ml), and amphotericin B (25 μ g/ml). Cultures were carried out at 37 °C in an atmosphere of 5% CO₂ in humidified air in six-well plates, 24-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) or 96-well plates (Corning Glass Works, Corning, NY, USA) for 48 h. The final amount of T-I cells plated was 700 $\times 10^3$ cells/2 ml in six-well plates, 350 $\times 10^3$ cells/ml in 24-well plates, and 35 $\times 10^3$ cells/0.25 ml in 96-well plates.

DNA synthesis determination

Radiolabeled [³H]thymidine (1 μ Ci/well) was added to cultured T-I cells during the last 24 h of culture. At the end of the culture period, the cells were harvested using a multiwell cell harvester (PHD Harvester-Model 290,

Cambridge Technology, Inc., Watertown, MA, USA). Radioactivity was measured in a liquid scintillation counter, SL 4000 (Intertechneque, Fairfield, NJ, USA).

Cell counting, identification of steroidogenically active cells

T-I cells were cultured in 24-well plates; each treatment was carried out at least in triplicates. At the end of the culture period, the cells were washed with PBS. Trypsin-EDTA (0.05% and 0.02% respectively; 0.3 ml/2 cm²) solution was dispensed into culture wells to completely cover the monolayer of cells and the culture dish was placed at 37 °C for 2–3 min. When the cells were in suspension and appeared rounded, McCoy's 5a medium was added to inhibit trypsin activity. Subsequently, the T-I cells were washed with PBS and fixed in 1% paraformaldehyde for 20 min. Steroidogenically active T-I cells were identified histochemically by detection of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity as described previously (Duleba *et al.* 1997). The number of stained cells (steroidogenically active) and non-stained cells (steroidogenically inactive) was determined using a hemocytometer by counting ten squares from each sample. Prior to cell counting, the samples were randomized and the observer counting the cells was blinded to the treatment.

Apoptosis studies

Apoptosis was studied using our serum withdrawal model: T-I cells were cultured for 24 h in six-well plates with 10% fetal bovine serum (attachment phase); subsequently, the cells were cultured for an additional 24 h with serum or in serum-free medium without growth factors (control), with TGF- α (10 ng/ml) and/or TGF- β (10 ng/ml). At the end of the 24 h period, the plates were washed with PBS, and total DNA was extracted as described by others (Gross-Bellard *et al.* 1973). Apoptosis was identified by detection of internucleosomal DNA cleavage producing the characteristic 'ladder pattern' of low-molecular weight (LMW) DNA following agarose gel electrophoresis. Quantification of apoptosis was carried out with the aid of 3'-end labeling of DNA fragments with [³²P]-dideoxy-ATP. Autoradiography was performed to determine the presence of internucleosomal DNA cleavage (Tilly *et al.* 1991).

Statistical analysis

Results are presented as the mean $\pm$ S.E.M. Comparisons between the means were performed using Kruskal-Wallis test followed by Mann-Whitney *U* test or, when appropriate, by ANOVA followed by *post hoc* comparisons of individual means using Bonferroni correction. Differences were considered significant at $P < 0.05$.

Figure 1 Effects of TGF- α and TGF- β on proliferation. The cells were cultured in serum-free medium for 48 h in 96-well plates at a concentration of 35 000 cells/well. Each well contained 0.25 ml medium. During the last 24 h of culture, [³H]thymidine (1 μ Ci/well) was added to determine DNA synthesis. Bars indicate the mean of each treatment and vertical lines indicate the S.E.M. from at least six replicates; * denotes means significantly different from control ($P < 0.05$); † denotes means significantly different from TGF- α alone and TGF- β alone ($P < 0.05$).

Results

Effects of TGF- α and TGF- β on DNA synthesis

Figure 1 summarizes the effects of TGF- α and TGF- β on DNA synthesis by T-I cells. Both TGF- α and TGF- β significantly increased DNA synthesis at all tested doses. TGF- α at a dose of 1 ng/ml induced the maximum stimulation of DNA synthesis by 3.1-fold above control ($P < 0.001$). TGF- β increased DNA synthesis in a dose-dependent fashion with the greatest effect observed at a dose of 10 ng/ml resulting in a 3.4-fold increase above control ($P < 0.005$). A combination of TGF- α and TGF- β produced a dose-dependent synergistic increase in DNA synthesis by up to tenfold above control ($P < 0.001$) at a dose of 10 ng/ml of each TGF- α and TGF- β .

Determination of the number of steroidogenically active and inactive T-I cells

Figures 2 and 3 present the results of counting T-I cells at the end of the 48-h culture period. TGF- α (10 ng/ml) alone increased the total number of cells by 2.8-fold; similarly TGF- β (10 ng/ml) alone increased the total number of cells by 2.8-fold. The combination of TGF- α (10 ng/ml) and TGF- β (10 ng/ml) increased the total cell count by 3.2-fold, compared with control (Fig. 2). The proportion of steroidogenically active cells was comparable in control cultures and cultures treated with TGF- α and/or TGF- β (Fig. 3).

Figure 2 Effects of TGF- α and TGF- β on the total cell count. The cells were cultured in serum-free medium for 48 h in 24-well plates at a concentration of 350 000 cells/well. The cells were cultured without additives (control) or with TGF- α (10 ng/ml) and/or TGF- β (10 ng/ml). Bars indicate the mean of each treatment and vertical lines indicate the S.E.M. from at least four replicates; * denotes means significantly different from control ($P < 0.05$).

Effect of TGF- α and TGF- β on apoptosis

Presence of 10% serum protected T-I cells from apoptosis and reduced the 3'-end labeling of LMW DNA by $68 \pm 10\%$ ($P = 0.01$) compared with control cultures in the absence of serum. In the absence of serum, addition of TGF- α (10 ng/ml) decreased apoptosis by $63 \pm 14\%$ ($P = 0.02$), while addition of TGF- β (10 ng/ml) had no statistically significant effect. A combination of TGF- α and

Figure 4 Effects of TGF- α and TGF- β on apoptosis, as determined by internucleosomal DNA cleavage induced by serum withdrawal. The cells were plated in six-well plates at a concentration of 700 000 cells/well and pre-incubated for 24 h with 10% serum. Subsequently, the cells were re-incubated for 24 h with 10% serum, in serum-free medium (control), or in serum-free medium supplemented with TGF- α (10 ng/ml) and/or TGF- β (10 ng/ml). Apoptosis is expressed as percentage of internucleosomal cleavage detected in cultures with 10% serum. Bars indicate the mean of each treatment and vertical lines indicate the S.E.M. from at least three replicates; * denotes means significantly different from control ($P < 0.05$).

TGF- β produced the greatest inhibition of apoptosis by $73 \pm 8\%$ ($P = 0.01$). Effects of TGF- α and TGF- β on apoptosis are summarized in Fig. 4.

Discussion

The results of the present study demonstrate that TGF- α and TGF- β , alone and in combination, stimulate growth of cultured rat T-I cells. The growth of T-I cells is an effect of increased DNA synthesis and decreased apoptotic death resulting in an increase in the number of both steroidogenically active and inactive cells. The novelty of our observations is related in particular to the observations of the effects of TGF- β . To our knowledge, this is the first report documenting that this agent promotes growth of T-I cells. Furthermore, effects of TGF- β appear to be distinct and independent from those of TGF- α : this conclusion is based on the synergistic effects of these growth factors on DNA synthesis and the evidence that TGF- β predominantly stimulates cell proliferation, while TGF- α significantly increases proliferation and decreases apoptosis. These effects are observed at physiologic levels of TGFs. Indeed, TGF- α has been detected in follicular fluid from normal ovaries at levels ranging from 0.2 to 200 ng/ml (Mason *et al.* 1995); TGF- β has been secreted by rat T-I cells at levels ranging from 0.1 to 10 ng/ml (Magoffin *et al.* 1995). In view of these observations, our

Figure 3 Effects of TGF- α and TGF- β on the proportion of steroidogenically active cells. The cells were cultured as described in Fig. 2. Steroidogenically active cells were identified histochemically upon detection of 3 β -HSD activity. Bars indicate the mean of each treatment and vertical lines indicate the S.E.M. from at least four replicates.

findings suggest that physiologic concentrations of both TGF- α and TGF- β may play a role in the stimulation of the growth of ovarian mesenchyme during development and folliculogenesis.

In this study, three parameters of growth or regression of cell populations were evaluated: thymidine incorporation (as a marker of DNA synthesis), cell counting, and DNA fragmentation (as a marker of apoptosis). TGF- α and TGF- β , especially in combination, induced concordant effects on all these parameters and caused an increase of DNA synthesis, increase of the cell number, and a decrease of apoptosis. However, synergistic stimulation of DNA synthesis by a combination of TGF- α and TGF- β was associated with only a modest increase of the cell number; the explanation of this phenomenon may be due to the fact that the change of the cell number reflects the net effect of two processes: mitotic activity and apoptosis. In this study, the exact contribution of each of these processes to the cell number cannot be elucidated. For example, it is possible that an increase of the cell number and an increase of DNA synthesis may be due only to a decrease of apoptosis alone. In such a scenario, an increase of the cell survival can lead to a greater number of cells capable of incorporating thymidine without alteration of the mitotic activity of the cell. Irrespective of the relative contribution of mitotic activity and apoptosis, TGF- α and TGF- β increased the number of both steroidogenically active and inactive cells. Most likely, the cells classified in this study as steroidogenically active represent follicular theca, while the steroidogenically inactive cells represent stromal component of ovarian mesenchyme.

The observations of the present study may be relevant to the understanding of physiological and pathological aspects of the thecal and stromal growth. Under physiological conditions, TGFs may contribute to appropriate growth and subsequent maintenance of ovarian homeostasis, while excessive activity of these peptides may contribute to thecal and stromal hyperplasia, such as seen in patients with polycystic ovary syndrome (PCOS) or hyperthecosis. Indeed, a link between EGF/TGF- α and PCOS has previously been proposed, partly based on the observations of increased levels of EGF in the follicular fluid and higher levels of EGF receptors in granulosa cells from patients with PCOS (Volpe *et al.* 1991, Barreca *et al.* 1996). Furthermore, systemic administration of EGF to rats resulted in polycystic growth of the ovaries (Christiansen *et al.* 1996).

Previous studies on the effect of TGFs on growth of ovarian tissues yielded conflicting findings. Most reports focused on granulosa cells, in which effects of TGFs on proliferation seemed to be species-dependent. TGF- α (and/or EGF) stimulated proliferation in the majority of studied species including bovine, porcine, and human granulosa cells (Gospodarowicz & Bialecki 1979, May *et al.* 1988, Skinner & Coffey 1988), but had no effect on rat cells (Bendell & Dorrington 1990). In contrast, TGF- β

stimulated proliferation of rat granulosa cells (Dorrington *et al.* 1988), but had no mitogenic activity of cells from cow or pig (May *et al.* 1988, Skinner *et al.* 1987). In theca cells, TGF- α /EGF stimulated proliferation in hen, cow and sheep (Roberts & Skinner 1991, Onagbesan *et al.* 1994b, Campbell 1999), but had no effect on the cell number in rat (Foghi *et al.* 1997). TGF- β , on the other hand, suppressed thymidine incorporation by bovine theca (Roberts & Skinner 1991), but had no effect on cell number in bovine or rat thecal cultures (Roberts & Skinner 1991, Foghi *et al.* 1997). To our knowledge, only one group of researchers evaluated interactions between TGF- α and TGF- β on growth of thecal cells (Foghi *et al.* 1997, 1998); in these studies, at select concentrations of cells, TGF- α in combination with TGF- β decreased cell count and induced apoptosis (Foghi *et al.* 1997, 1998). Interestingly, in the same study, neither TGF- α alone, nor TGF- β alone, had any detectable effect on cell count or apoptosis (Foghi *et al.* 1997). These observations contrast with the present findings, whereby both TGF- α and TGF- β promoted growth of T-I cells and synergistically stimulated DNA synthesis. The discrepancy between the present observations and those of Foghi *et al.* may be due to the difference in the animal models. In our study, T-I cells were obtained from estradiol-treated Sprague-Dawley rats. Estrogen priming has been shown to induce follicle-stimulating hormone (FSH) receptors and decrease ovarian androgen production (Louvet & Vaitukaitis 1976, Leung & Armstrong 1979), possibly reflecting decreased follicular atresia. Furthermore, in several experimental models, estrogen has been shown to modulate expression of EGF/TGF- α and TGF- β receptors (De Bortoli & Dati 1997, Chang *et al.* 1999, Salvatori *et al.* 2000). However, it is unlikely that the change in the expression of TGF receptors could account for the contrasting observations in our studies and the studies of Foghi *et al.* A more probable explanation may relate to the fact that Foghi *et al.* used cells from 25-day-old untreated Wistar rats; in that model, the ovary exhibits a wave of atresia of preantral follicles (Foghi *et al.* 1997).

Review of the literature reveals highly variable effects of interactions of TGF- α and TGF- β on growth. Thus, in bovine granulosa cell cultures, TGF- β inhibited stimulatory effect of EGF on growth (Skinner *et al.* 1987) but in porcine granulosa cells TGF- β potentiated EGF's stimulatory effect on cell division (May *et al.* 1988). Outgrowth of chicken erythrocytic progenitor cells from various sources invariably required both TGF- α and TGF- β (Gandrillon *et al.* 1999). Furthermore, TGF- α and TGF- β cooperatively stimulated proliferation of human malignant glioma cells (Kurimoto *et al.* 1994). In contrast, in rabbit gastric epithelial cells, co-administration of TGF- β abolished EGF-stimulated DNA synthesis (Nakajima & Kuwayama 1993). Thus, it appears that the effects of a combination of TGF- α and TGF- β are both species- and tissues-dependent.

In summary, this study demonstrates that TGF- α and TGF- β , alone and in combination, increase the number of T-I cells by increasing their mitotic activity and/or decreasing apoptosis. Availability of and responsiveness to growth factors such as TGFs may be important to both physiologic events including ovarian development and folliculogenesis, as well as pathological conditions such as PCOS, whereby excessive growth of ovarian mesenchyme may be the underlying cause of ovarian dysfunction.

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