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Estrogens Modulate the Inhibitory Effect of Tumor Necrosis Factor-α on Anterior Pituitary Cell Proliferation and Prolactin Release

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Considering that tumor necrosis factor-α (TNF-α) is involved in normal tissue homeostasis and that its receptors are expressed in the anterior pituitary, we examined the effect of this cytokine on pituitary cell growth. Because anterior pituitary function depends on hormonal environment, we also investigated the influence of gonadal steroids in the effects of TNF-α on cell proliferation and the release of PRL from anterior pituitary cells. In addition, the release of TNF-α and its action on the release of PRL from anterior pituitary cells of rats at different stages of the estrous cycle was evaluated. In minimum essential medium D-valine, a medium that restricts fibroblastic proliferation, TNF-α (10 and 50 ng/mL) reduced ^{3}H-Thymidine incorporation, DNA content, and active cell number. TNF-α failed to affect proliferation of cells from ovariectomized (OVX) rats. However, it significantly inhibited growth of cells from OVX rats cultured with 17β-estradiol (E_2) (10^{-9} *M*) and from chronically estrogenized rats. TNF-α decreased the release of PRL from cells of intact rats, especially in proestrous, OVX rats cultured with E_2 and chronically estrogenized rats. The release of anterior pituitary TNF-α was higher in proestrous rats. These results indicate that TNF-α plays an inhibitory role in anterior pituitary cell growth and the release of PRL in an estrogen-dependent manner.

Key Words: Tumor necrosis factor-α; anterior pituitary; prolactin; growth; estrogens; estrous cycle.

Introduction

Anterior pituitary hormone secretion is regulated by signals from the hypothalamus and the neurointermediate lobe, as well as by hormones produced in peripheral tissues *(1,2)*. Also, locally synthesized peptides, acting in an autocrine or paracrine manner, regulate hormone release from the anterior pituitary. Several cytokines and growth factors are expressed in the anterior pituitary, where they have a modulatory role in secretory and proliferative functions *(3,4)*.

Tumor necrosis factor-α (TNF-α), a pleiotropic cytokine produced by several cell types such as activated macrophages and microglia, was originally identified as a systemic mediator of endotoxemic shock, cachexia, and tumor regression *(5)*. TNF-α is synthesized and released by the brain and anterior pituitary *(6–8)*, where its receptors are also expressed *(9)*, suggesting that TNF-α may participate in the control of neuroendocrine functions. However, reports concerning direct effects of TNF-α on pituitary hormone secretion are controversial. Regarding prolactin (PRL), it has been observed that TNF-α had no effect *(10)* or stimulated the release of PRL after short incubation periods *(11,12)*, whereas it inhibited basal and thyrotropin-releasing hormone–induced release of PRL after long incubation periods *(13,14)*.

TNF-α is involved in the regulation of normal tissue homeostasis affecting cell proliferation, differentiation, and apoptosis *(15,16)*. It has been shown that TNF-α exerts both stimulatory and inhibitory effects on cell proliferation, depending on the target cell. TNF-α stimulates the proliferation of fibroblasts, ovarian granulosa cells, and hepatocytes *(17–19)*. By contrast, this cytokine has an inhibitory effect on the growth of some normal cell types *(20,21)*, as well as on several human tumor cell lines *(22,23)*.

Anterior pituitary cell proliferation, differentiation, and function depend on hormonal status *(24,25)*. The direct effects of estrogens on the anterior pituitary are exerted when they bind to specific receptors localized in lactotrophs, gonadotrophs, corticotrophs, and folliculo-stellate cells *(26)*. Estrogen receptor activates PRL gene transcription and is involved as a lactotroph growth-stimulating factor *(27)*. The estrogen-induced upregulation of pituitary growth factor gene expression, as was reported for transforming growth factor-α and fibroblast growth factor-2 *(3)*, suggests that estrogens could act on anterior pituitary cell growth by

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affecting the synthesis of autocrine/paracrine factors. We have previously reported that estrogens stimulate the release of TNF-α from anterior pituitary cells *(7)*.

The aim of the present study was to explore the role of TNF-α on anterior pituitary growth. We examined the effects of TNF-α on proliferation of anterior pituitary cells from female rats at random stages of the estrous cycle and compared its effects with those of interleukin-6 (IL-6). To evaluate whether hormonal environment affects the activity of TNF-α in the anterior pituitary, we investigated the influence of gonadal steroids in the effects of TNF-α on anterior pituitary cell proliferation and PRL secretion. Release of TNF-α from anterior pituitary cells of rats at selected stages of the estrous cycle was also studied.

Results

Effect of TNF-α and IL-6 on Proliferation of Anterior Pituitary Cells from Intact Rats

When the anterior pituitary cells of pooled intact rats were cultured in Dulbecco's modified Eagle's medium (DMEM), TNF-α significantly stimulated ^{3}H-Thymidine incorporation at concentrations above 50 ng/mL (Fig. 1). TNF-α (50 ng/mL, approx 3×10^{-9} *M*) also increased DNA content and active cell number (by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] assay) (Table 1). IL-6 (1 ng/mL) did not modify ^{3}H-Thymidine incorporation, DNA concentration, or active cell number (Table 1).

Because fibroblastic proliferation is stimulated by TNF-α *(23)*, we studied its effect on ^{3}H-Thymidine incorporation by anterior pituitary cells cultured in minimum essential medium Eagle (MEM-D-valine), a medium in which fibroblastic growth is abrogated *(28)*. In contrast to the stimulatory effect of TNF-α on cells cultured in DMEM, TNF-α (10 and 50 ng/mL) significantly inhibited ^{3}H-Thymidine incorporation (Fig. 2). In MEM-D-valine, TNF-α and IL-6 also reduced DNA content and active cell number (Table 2). These results showed that the inhibitory action of TNF-α on the proliferation of anterior pituitary secretory cells could be unmasked when cells were cultured in MEM-D-valine. Therefore, all the following experiments on cell proliferation were performed in this medium.

Effect of Ovarian Steroids on Antiproliferative Action of TNF-α in Ovariectomized Rats

To evaluate the influence of gonadal steroids in the effect of TNF-α on cellular proliferation, we investigated the effect of TNF-α on ^{3}H-Thymidine incorporation by anterior pituitary cells from ovariectomized (OVX) rats in the presence of 17β-estradiol (E_2) or progesterone. TNF-α did not significantly modify proliferation of cells from OVX rats, whereas it significantly inhibited ^{3}H-Thymidine incorporation in the presence of E_2 (10^{-8} *M*) (Fig. 3).

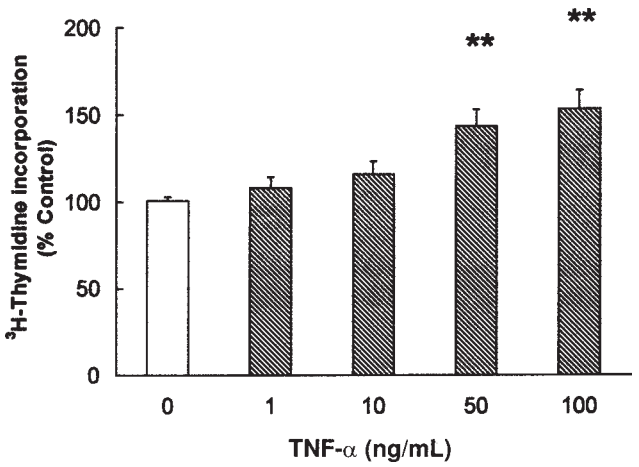

Fig. 1. Effect of TNF-α on growth of anterior pituitary cells from intact rats. Cells were cultured for 3 d in DMEM-S (2.5% FBS), 1 d in DMEM-S without FBS, and then 2 d in the same fresh medium containing ^{3}H-Thymidine (1 μCi/mL) and TNF-α. The basal control value was 4966.6 ± 267.8 dpm/well. Each column represents the mean ± SE of 4–10 wells. Data were analyzed by analysis of variance (ANOVA) followed by Dunnett´s test. $^{**}p < 0.01$ vs control.

TNF-α did not affect ^{3}H-Thymidine incorporation to cells from OVX rats cultured in the presence of progesterone (10^{-6} *M*) (data not shown). In an additional experiment, we evaluated the effect of TNF-α on cell growth of anterior pituitary cells from OVX chronically estrogenized rats. In these cell cultures, TNF-α markedly inhibited ^{3}H-Thymidine incorporation (control: 3234.5 ± 144.6 dpm/well; TNF-α: 2197.2 ± 136.4; $p < 0.01$; $n = 8$).

Effect of TNF-α on Prolactin Release

TNF-α decreased the release of PRL from anterior pituitary cells of rats at random stages of the estrous cycle (Fig. 4). TNF-α also inhibited the release of PRL from anterior pituitary cells of rats in proestrus and estrus but not in diestrus (Fig. 5). E_2 (10^{-9} *M*) induced a significant increase in the release of PRL from cells of OVX rats. TNF-α significantly reduced the stimulatory effect of E_2 on the release of PRL (Fig. 6). TNF-α also decreased the release of PRL from anterior pituitary cells of chronically estrogenized rats (control: 3.32 ± 0.27 μg/mL; TNF-α: 1.65 ± 0.11; $p < 0.01$; $n = 8$).

Release of TNF-α During Estrous Cycle

When the release of TNF-α from anterior pituitary cells of rats killed at different stages of the estrous cycle was evaluated, the highest release of TNF-α was observed in cells of proestrous rats (Fig. 7).

Discussion

The present study shows that TNF-α is involved as an inhibitory factor in the regulation of anterior pituitary cell growth. The antiproliferative effect of TNF-α on several

Table 1
Effect of TNF-α and IL-6 on Anterior Pituitary Cell Growth in DMEM[a]

	^{3}H-thymidine (dpm/well)	DNA (ng/well)	Active cell number (OD)
Control	2497.8 ± 128.4	206.0 ± 12.1	0.273 ± 0.010
TNF-α (50 ng/mL)	3217.4 ± 210.1[b]	292.0 ± 16.8[c]	0.358 ± 0.010[c]
IL-6 (1 ng/mL)	2651.9 ± 180.9	234.0 ± 11.9	0.261 ± 0.004

[a] Anterior pituitary cells from rats at random stages of the estrous cycle were cultured for 3 d in DMEM-S (2.5% FBS), 1 d in DMEM-S without FBS, and then for 2 d in the same fresh medium containing TNF-α or IL-6 in the presence of ^{3}H-Thymidine (1 μCi/mL) when relevant. Each value represents the mean ± SE of five to eight wells. Data were analyzed by ANOVA followed by Dunnett´s test. OD, optical density. [b] $p < 0.05$; [c] $p < 0.01$ vs respective control.

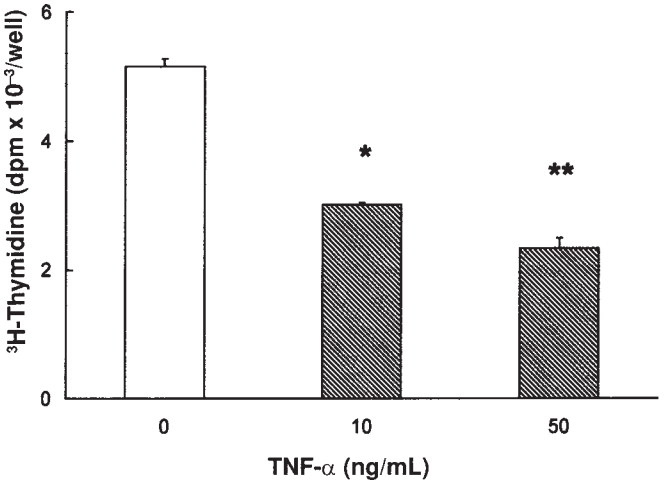

Fig. 2. Effect of TNF-α on growth of anterior pituitary cells from intact rats cultured in MEM-D-valine. Cells were cultured for 3 d in MEM-D-valine-S (10% FBS), 1 d in MEM-D-valine-SS without FBS, and then 2 d in the same fresh medium containing ^{3}H-Thymidine (1 μCi/mL) and TNF-α. Each column represents the mean ± SE of five to seven wells. Data were analyzed by ANOVA followed by Dunnett´s test. $^*p < 0.05$; $^{**}p < 0.01$ vs control.

normal and tumoral cell types is well documented *(20–23)*. The inhibitory effect of TNF-α on cell growth could result from action on the G1/S transition of the cell cycle or by triggering apoptotic mechanisms *(16,29)*. It is unlikely that the antiproliferative effect of TNF-α on anterior pituitary cells results from cytotoxicity because it has been reported that TNF-α has no cytotoxic action on anterior pituitary cells cultured under conditions similar to ours *(13,30)*. It has already been reported that IL-6 decreased ^{3}H-Thymidine incorporation to anterior pituitary cells cultured in serum-free MEM-D-valine, a medium that abrogates fibroblastic proliferation *(28)*, suggesting that this cytokine inhibits the proliferation of secretory cells *(31)*. In this medium, TNF-α reduced ^{3}H-Thymidine incorporation, DNA content, and the number of active pituitary cells, indicating that TNF-α may specifically inhibit the proliferation of secretory cells as was suggested for IL-6 *(31)* and supported by our data. Because lactotrophs account for the highest proportion of proliferative cells in the anterior pituitary *(32)*, it is possible to suggest that

Table 2
Effect of TNF-α and IL-6 on Anterior Pituitary Cell Growth in MEM-D-valine[a]

	DNA (ng/well)	Active cell number (OD)
Control	338.5 ± 6.6	0.266 ± 0.010
TNF-α (10 ng/mL)	268.9 ± 3.7[c]	0.237 ± 0.010[b]
TNF-α (50 ng/mL)	266.4 ± 5.7[c]	0.209 ± 0.005[c]
IL-6 (1 ng/mL)	296.4 ± 9.0[c]	0.233 ± 0.006[b]

[a] Anterior pituitary cells from rats at random stages of the estrous cycle were cultured for 3 d in MEM-D-valine-S (10% FBS), 1 d in MEM-D-valine-SS without FBS, and then for 2 d in the same fresh medium containing TNF-α or IL-6. Each value represents the mean ± SE of six to eight wells. Data were analyzed by ANOVA followed by Dunnett´s test. [c] $p < 0.05$; [c] $p < 0.01$ vs respective control.

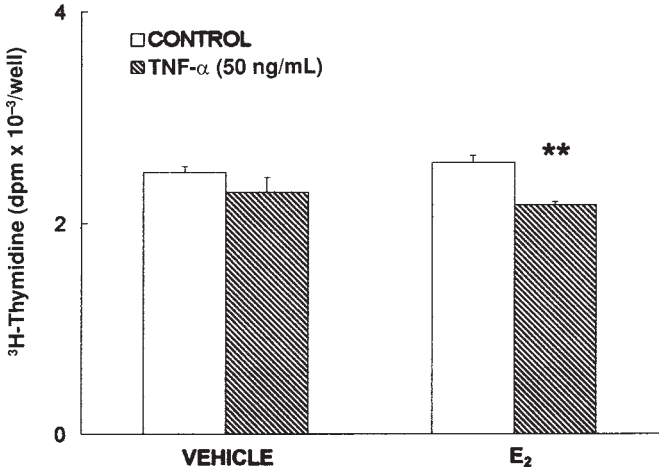

Fig. 3. Effect of TNF-α on growth of anterior pituitary cells from OVX rats in the presence of E_2. Cells were cultured for 3 d in MEM-D-valine-S (10% FBS-DCC), 1 d in MEM-D-valine-SS without FBS, and then for 2 d in the same medium containing E_2 (10^{-9} *M*) or vehicle (0.1 μL/mL of ethanol). Finally, cells were incubated for another 2 d in the same fresh medium containing ^{3}H-Thymidine (1 μCi/mL) and TNF-α. Each column represents the mean ± SE of six wells. Data were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. $^{**}p < 0.01$ vs respective control without TNF-α.

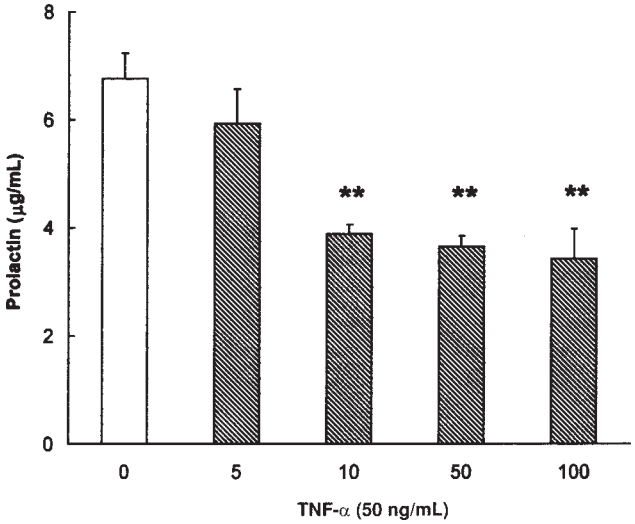

Fig. 4. Effect of TNF-α on the release of PRL from anterior pituitary cells of intact rats. Cells were cultured for 3 d in DMEM-S (10% fetal bovine serum [FBS]), 1 d in DMEM-S (0.1% bovine serum albumin [BSA]) without FBS, and then for 2 d in the same fresh medium with TNF-α. Each column represents the mean ± SE of five to six wells. Data were analyzed by ANOVA followed by Dunnett´s test. $^{**}p < 0.01$ vs respective control without TNF-α.

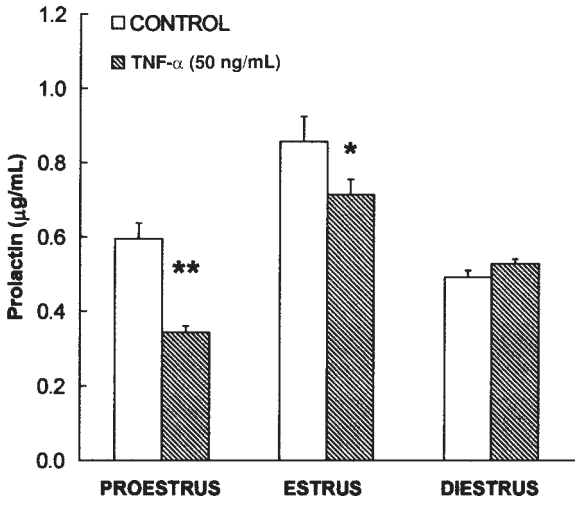

Fig. 5. Effect of TNF-α on the release of PRL from anterior pituitary cells of rats at selected stages of the estrous cycle. Cells from three to four rats sacrificed at each stage of the estrous cycle were cultured for 3 d in DMEM-S (10% FBS), 1 d in DMEM-S (0.1% BSA) without FBS, and 8 h in the same fresh medium with TNF-α. Each column represents the mean ± SE of 9–10 wells. Data were analyzed by Student´s *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$ vs respective control without TNF-α.

both TNF-α and IL-6 may affect the proliferation of this subpopulation of pituitary secretory cells. However, because folliculo-stellate cells can also grow in a medium lacking L-valine (data not shown), the action of TNF-α on the proliferation of folliculo-stellate cells and other secretory cells different from lactotrophs cannot be discarded.

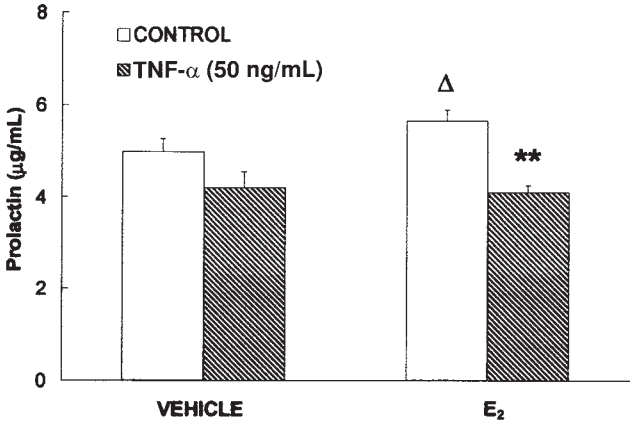

Fig. 6. Effect of TNF-α on the release of PRL from anterior pituitary cells of OVX rats in the presence of E_2. Cells were cultured for 3 d in DMEM-S (10% FBS-DCC), 1 d in DMEM-S (0.1% BSA) without FBS, and 2 d in the presence of E_2 (10^{-9} *M*) or vehicle (0.1 μL/mL of ethanol). Finally, cells were incubated for another 2 d in the same fresh medium with TNF-α. Each column represents the mean ± SE of nine wells. Data were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. $^{**}p < 0.01$ vs respective control without TNF-α; $^{\Delta}p < 0.05$ vs respective control without E_2.

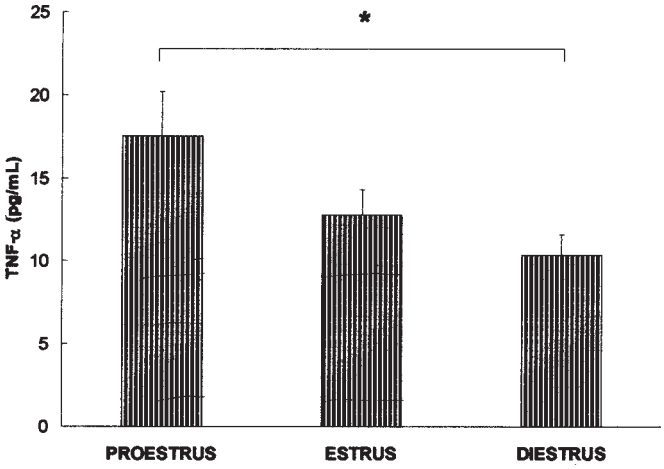

Fig. 7. TNF-α release from anterior pituitary cells of rats at selected stages of the estrous cycle. Cells were cultured for 3 d in DMEM-S (10% FBS), 1 d in DMEM-S (0.1 % BSA) without FBS, and then incubated for 8 h in the same medium. Each column represents the mean ± SE of six to seven wells. Data were analyzed by ANOVA followed by Student-Newman-Keuls test. $^{*}p < 0.05$ vs proestrus.

Homeostatic control of cell number is thought to be the result of the dynamic balance between cell proliferation and cell death. Apoptosis is a physiological process that plays a basic role in the regulation of cell populations including normal and tumorous pituitary cells *(33–35)*. Moreover, it has been suggested that small fluctuations in mitotic and apoptotic events have profound effects on pituitary cell population dynamics *(35)*. It has been reported that lactotroph apoptosis is enhanced at the end of lactation

and with estrogen withdrawal *(33,36)*. The enhanced inhibitory effect of TNF-α on cell growth in the presence of estradiol indicates that this action is modulated by estrogens. The release of TNF-α varied during the estrous cycle, reaching the highest levels in proestrus. This same pattern for TNF-α production has been described in the mouse uterus, where gonadal steroids regulate the synthesis of TNF-α *(37,38)*. Furthermore, we have previously reported that estrogens stimulate the release of TNF-α from rat anterior pituitary cells *(7)*. Therefore, the high level of estrogens during proestrus may participate in the stimulation of the release of TNF-α during this estrous cycle stage. Apoptosis in steroid-dependent tissues can be initiated by the direct action of the steroids on target cells or indirectly, by altering the expression of paracrine effectors such as TNF-α *(39)*. It has been suggested that TNF-α is involved in cyclic apoptotic events in the endometrial epithelial cells *(40,41)*. The rise in the release of TNF-α in proestrus, together with the impact of TNF-α on cell proliferation, suggests that this cytokine could participate as a paracrine/autocrine factor in the regulation of anterior pituitary cell renewal.

The reports concerning the action of TNF-α on PRL secretion are controversial *(10–14)*, and the discrepancies may result from differences in experimental conditions, especially regarding the use of hemipituitaries or dispersed cells, cell density, and duration of exposure to the cytokine. Under the present experimental conditions, TNF-α inhibited PRL secretion from cells of pooled intact rats, as was previously shown after an 8-h incubation period *(7)*. The presence of TNF-α resulted in a reduced release of PRL from cells of proestrous and estrous rats but not from diestrous rats. Because the inhibitory effect of TNF-α on the release of PRL from cells of OVX rats was observed only in the presence of E_2, it seems that estrogens have a permissive role in the response of PRL to TNF-α. Our data suggest that the gonadal steroid environment may influence TNF-α release and activity in the anterior pituitary during the estrous cycle.

Several cytokines and growth factors are locally produced in the anterior pituitary. They may control secretory activity as well as differentiation and growth of anterior pituitary cells, acting directly or indirectly by affecting other intrinsically synthesized molecules in the anterior pituitary *(4)*. There is strong evidence that some cytokines and growth factors expressed in the pituitary affect cell proliferation and may be involved in the process of tumor formation in this gland *(4,27)*. The expression of TNF-α has been documented in rat pituitary cells as well as in human pituitary adenomas *(8,9,42)*. Because cell growth could result from either stimulated proliferation or deficient cell arrest, the involvement of TNF-α in the regulation of pituitary cell proliferation raises the possibility that alterations in the activity of this cytokine may play a role in pituitary tumor initiation or maintenance.

In summary, we have demonstrated that TNF-α is an inhibitory growth factor for normal rat pituitary cells. Our data also provide evidence that the activity of TNF-α on pituitary cell proliferation and PRL secretion is estrogen dependent. Considering that TNF-α and its receptors are expressed in pituitary cells, this cytokine may participate as an autocrine/paracrine modulator of anterior pituitary function. In addition, TNF-α could also be involved in the regulation of pituitary growth and secretion under pathological conditions such as endotoxemia or pituitary tumorigenesis.

Materials and Methods

All drugs, media and supplements were obtained from Sigma (St. Louis, MO), except FBS (GenSa, Buenos Aires, Argentina), rhTNF-α and rhIL-6 (Promega, Madison, WI), and the materials indicated in the following sections.

Animals

Adult female Wistar rats were kept under controlled conditions of light (12 h light/dark cycles) and temperature (20–25°C). Rats were fed with standard lab chow and water ad libitum and maintained in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

Intact rats were killed by decapitation at random stages of the estrous cycle or monitored by daily vaginal smears during three consecutive cycles and killed in estrus, diestrus, or proestrus. A group of rats was OVX under ether anesthesia, and immediately thereafter some were implanted subcutaneously with Silastic capsules containing 1 mg of E_2. Animals were killed by decapitation 2 wk after surgery.

Cell Culture

Anterior pituitary glands (neurointermediate lobe removed) were obtained within minutes after decapitation. The glands were washed several times with DMEM and cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in DMEM supplemented with 3 mg/mL of BSA containing 5 mg/mL of trypsin (Type XII-S from bovine pancreas), 1 mg/mL of DNase (deoxyribonuclease II, type V from bovine spleen), and 1 mg/mL of trypsin inhibitor (type II-S from soybean), and finally were dispersed by extrusion through a Pasteur pipet in Krebs buffer without Ca^{2+} and Mg^{2+}.

Dispersed cells were washed twice and suspended either in DMEM supplemented with 10 μL/mL of MEM amino acids, 2 m*M* glutamine, 5.6 μg/mL of amphotericin B, and 25 μg/mL of gentamicin (DMEM-S) or in MEM-D-valine containing D-valine instead of L-valine, supplemented with 10 μL/mL of MEM nonessential amino acids, 2 m*M* glutamine, and antibiotics (MEM-D-valine-S). Cell viability as assessed by trypan blue exclusion was above 90%. The cells were seeded either onto 48-well tissue culture plates (25×10^4 cells/0.5mL/well) for ^{3}H-Thymidine incorporation or DNA content or onto 96-well tissue culture plates (8×10^4 cells/0.25mL/well) for determination of active cell number and release of PRL or TNF-α. The cells were

cultured for 3 d (37°C, 5% CO_2 in air) in DMEM-S with 2.5% FBS or in MEM-D-valine-S with 10% FBS. After the culture period, the cells were washed twice and the media replaced with serum-free DMEM-S or MEM-D-valine-S supplemented with 10 μg/mL of insulin, 20 n*M* sodium selenium, 5 μg/mL of transferrin, 0.02 ng/mL of triiodothyronin, and 10 μL/mL of MEM vitamins (MEM-D-valine-SS). The cells were preincubated in these media for 1 d to wash out the remaining FBS and then incubated for 2 d with TNF-α or IL-6 in DMEM-S or MEM-D-valine-SS for ^{3}H-Thymidine incorporation, DNA content, and determination of active cell number, or 8 or 48 h in DMEM-S (0.1% BSA) for release of PRL and TNF-α. In the case of OVX chronically estrogenized rats, cells were processed as already indicated except that FBS was treated previously with 0.025% dextran-0.25% charcoal (FBS-DCC) in order to remove free steroids.

To study the direct influence of gonadal steroids on the effect of TNF-α, cells from OVX rats were dispersed as previously described and cultured in MEM-D-valine-S with 10% FBS-DCC. After the culture period, cells were washed twice, preincubated for 1 d in MEM-D-valine-SS without serum, and then incubated for 2 d in the presence of 10^{-9} *M* E_2, 10^{-6} *M* progesterone, or with vehicle (final ethanol concentration: 0.1 μL/mL). Finally, the cells were incubated for another 2 d in the same fresh medium containing the steroids and TNF-α. Release of PRL was determined under the same conditions except that cells were cultured in DMEM-S 10% FBS-DCC and incubated in DMEM-S (0.1% BSA) instead of MEM-D-valine-SS.

^{3}H-Thymidine Incorporation

During the last 48 h of culture, 1 μCi/mL of ^{3}H-Thymidine (New England Nuclear, Boston, MA; specific activity: >10 Ci/mmol) was added. After the incubation period, cells were washed twice with DMEM or MEM-D-valine, treated with 400 μL of Krebs buffer (Ca^{2+} and Mg^{2+} free) containing 0.25% trypsin for 20 min at 37°C, precipitated in 20% trichloroacetic acid (TCA), and then washed with 10% TCA. The material was centrifuged and the pellet dissolved in 100 μL of Solvable (New England Nuclear) and mixed with 1 mL of scintillation liquid (Optiphase Hisafe, Wallac, Turku, Finland). Radioactivity was measured in a liquid scintillation counter.

DNA Content

DNA content was measured by a fluorometric assay with bisbenzimidazole reagent *(43)*. Briefly, after the incubation period, cells were washed twice and sonicated in 10 m*M* Tris-HCL, 1 m*M* EDTA, and 0.1 *M* NaCl, pH 7.4. An aliquot of the sonicated solution was mixed with 0.01% bisbenzimidazole reagent dissolved in the same buffer. Fluorescence was determined in a fluorometer at a wavelength of 365 nm of excitation and 460 nm of emission.

MTT Assay

The MTT assay was used for quantitative determination of the number of active cells. This assay detects the net result in maintenance and proliferation of a cell population *(44)*. In brief, cells were washed twice and incubated for 4 h in 100 μL of Krebs buffer plus 50 μg of MTT reagent dissolved in 10 μL of phosphate-buffered saline (PBS) at 37°C. The developed crystals were dissolved in 100 μL of 0.04 *N* HCl in isopropanol, and the OD was read in a microplate spectrophotometer at a wavelength of 600 nm.

PRL Determination

PRL was measured by a double antibody radioimmunoassay with reagents provided by the National Hormone and Pituitary Program (Torrance, CA). RP-3 was used as the reference preparation and NIDDK-anti-rPRL-S-9 as the antiserum. The intra- and interassay coefficients of variation were <9%.

TNF-α Assay

TNF-α was determined by a specific rat TNF-α enzyme immunoassay (Cytoscreen Immunoassay Kit, Biosource, Camarillo, CA). The rat TNF-α antibody of this assay crossreacts with mouse TNF-α (100%) and human recombinant TNF-α (0.15%). The sensitivity of the assay was <4 pg/mL.

Statistical Analysis

Data were expressed as mean ± SE and evaluated by Student's *t*-test when two experimental groups were compared and one-way analysis of variance (ANOVA) when three or more groups were compared, followed by Dunnett's test for several comparisons against a single control group or by the Student-Newman-Keuls test for multiple comparisons. In two-factor interaction experiments, data were evaluated by two-way ANOVA followed by the Student-Newman-Keuls test. Differences between means were considered significant if $p < 0.05$. All experiments were performed at least twice. Results from individual experiments are presented in Figs. 1–7.

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