0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(9):3611–3617

doi: 10.1210/en.2002-220377

TNF-α Induces Apoptosis of Lactotropes from Female Rats

M. CANDOLFI, V. ZALDIVAR, A. DE LAURENTIIS, G. JAITA, D. PISERA, AND A. SEILICOVICH

Centro de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Buenos Aires 1121, Argentina

TNF-α is involved in the regulation of normal tissue homeostasis affecting cell proliferation, differentiation, and death. We previously reported that TNF-α reduces anterior pituitary cell proliferation and PRL release in an estrogen-dependent manner. In the present project we studied the induction of apoptosis by TNF-α in anterior pituitary cells from female rats. TNF-α (50 ng/ml) decreased the viability of anterior pituitary cells. Incubation with TNF-α for 24 h increased the percentage of terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling-positive cells. TNF-α increased the percentage of somatotropes and lactotropes with apoptotic nuclear morphology without affecting the proportion of apoptotic corticotropes or gonadotropes. TNF-α increased the percentage of apoptotic lactotropes in cultured cells from rats killed in proestrus and estrus, but not in diestrus. This effect was significantly higher in cells from rats in proestrus than in estrus. In anterior pituitary cells from ovariectomized rats, TNF-α significantly increased the percentage of apoptotic lactotropes only when the cells were incubated in the presence of 17β-estradiol. These results indicate that TNF-α induces apoptosis in somatotropes and lactotropes from female rats. The apoptotic effect of TNF-α on lactotropes is dependent on estrogens and could be involved in the regulation of anterior pituitary cell renewal during the estrous cycle. (*Endocrinology* 143: 3611–3617, 2002)

TNF-α IS A multifunctional cytokine with a central role in host defense (1). It elicits many biological activities, including cell proliferation, differentiation, and death (2). Low levels of TNF-α are found in several tissues, but its expression is induced by infection or other stressors (3–5). TNF-α is synthesized and released by the brain and pituitary (3–6). In the pituitary, TNF-α has been detected in somatotropes and neurointermediate lobe (7). Its receptors are also expressed in this gland (8, 9), suggesting that TNF-α may participate in the control of anterior pituitary function. In fact, this cytokine has been shown to affect the secretion of ACTH, GH, TSH, and PRL (10, 11). We previously reported that TNF-α reduces anterior pituitary cell proliferation and PRL release (12).

TNF-α acts through two types of specific receptors: TNF receptor 1 (TNFR-1) of 55 kDa and TNFR-2 of 75 kDa (13). Signaling by TNF-α, initiated by binding to TNFR-1, induces the association of adapter proteins to form a death-inducing signaling complex leading to apoptosis (14). These complexes provoke a proteolytic cascade that enables cell disassembly (15). Apoptosis can also be produced by a mechanism involving the release of cytochrome *c* and other proapoptotic proteins from mitochondria and subsequent activation of procaspase 9 (16). TNF-α also activates transcription factors such as nuclear factor-κB and activating protein-1, which may override the apoptotic pathways in some cells (2, 14, 17, 18).

Death by apoptosis is known to have a major role in the maintenance of tissue homeostasis. Apoptosis occurs in both normal and tumoral anterior pituitary cells (19–21). Evidence shows that lactotrope apoptosis occurring after termination of lactation involves changes in the expression of modulators of cell survival such as Bcl-2, Bax, and p53 (22).

In estrogen-dependent tissues, steroids can induce apoptosis directly or indirectly by modifying the expression of certain paracrine factors, including TNF-α (23). Gonadal steroids regulate TNF-α synthesis in the endometrium, where the highest expression of this cytokine occurs at midcycle (24, 25). We reported that estrogens stimulate TNF-α release from anterior pituitary cells and that this release is higher in cells from rats killed in proestrus (6, 12). We also observed that the inhibitory effect of TNF-α on anterior pituitary cell proliferation was estrogen dependent (12).

As TNF-α may be involved in anterior pituitary cell renewal, the aim of the present study was to explore whether TNF-α, besides inhibiting cell proliferation, promotes apoptosis in anterior pituitary cells and whether the apoptotic effect of TNF-α is dependent on estrogens. Therefore, we examined the effect of TNF-α on the viability and apoptosis of anterior pituitary cells from intact rats killed at random or at selected stages of the estrous cycle or from ovariectomized rats cultured in the presence of 17β-estradiol. We observed that TNF-α induced apoptosis of somatotropes and lactotropes. The promotion of apoptosis of lactotropes by TNF-α was predominant in cells from rats at proestrus and appears to be estrogen dependent.

Abbreviations: ER, Estrogen receptor; FBS, fetal bovine serum; FBS-DCC, dextran-charcoal-treated fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OVX, ovariectomized; TE, Tris-EDTA; TERP-1, truncated estrogen receptor product-1; TNFR, TNF receptor; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling.

Materials and Methods

All drugs, media, and supplements were obtained from Sigma (St. Louis, MO), except fetal bovine serum (FBS; GenSa, Buenos Aires, Argentina), recombinant human TNF-α (Promega Corp., Madison, WI), all terminal deoxynucleotidyltransferase-mediated deoxyuridine triphos-

phate nick end labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), and the materials indicated below.

Animals

Adult female Wistar rats were kept in controlled conditions of light (12-h light, 12-h dark cycles) and temperature (20–25 C). Rats were fed standard laboratory chow and water *ad libitum* and were kept 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 were monitored by daily vaginal smears over three consecutive cycles and killed in proestrus, estrus, or diestrus. Groups of rats were ovariectomized (OVX) with ether anesthesia 2 wk before the experiments.

Cell culture

Anterior pituitary glands (without neurointermediate lobe) were removed 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 BSA, containing 2.5 mg/ml trypsin (type I from bovine pancreas), 1 mg/ml deoxyribonuclease II (type V from bovine spleen), and 1 mg/ml trypsin inhibitor (type II-S from soybean) and were finally dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca^{2+} and Mg^{2+}. Dispersed cells were washed twice and resuspended in MEM containing D-valine instead of L-valine to abrogate fibroblast growth, supplemented with 5 μl/ml MEM nonessential amino acids, 2 mM glutamine, 5.6 μg/ml amphotericin B, and 25 μg/ml gentamicin (MEM-D-valine-S). Cell viability, as assessed by trypan blue exclusion, was greater than 90%. The cells were seeded onto coverslides in 24-well tissue culture plates (10×10^4 cells/0.5 ml/well) for the TUNEL method, immunocytochemistry, and nuclear microscopic observation, onto 96-well tissue culture plates (75×10^4 cells/0.20 ml/well) for metabolic activity of viable cell determination, or onto 6-well tissue culture plates (3.0×10^6 cells/4 ml/well) for DNA fragmentation analysis by electrophoresis. The cells were cultured for 2–3 d (37 C, 5% CO_2 in air) in MEM-D-valine-S with 10% FBS. After this period, cells were washed twice, and the medium was replaced by MEM-D-valine-S supplemented with 10 μg/ml insulin, 6.7 ng/ml sodium selenium, 5.5 μg/ml transferrin, 0.02 ng/ml T_3, and 10 μl/ml MEM vitamins (MEM-D-valine-SS) without FBS. The cells were cultured in this medium for 1 d to wash out remaining FBS and then incubated for 1 d in the same fresh medium with TNF-α (50 ng/ml). In the case of rats killed at selected stages of the estrous cycle, cells were processed as indicated above, except that FBS was treated previously with 0.025% dextran-0.25% charcoal (FBS-DCC) to remove steroids, and the wash-out period without serum was omitted.

In the case of OVX rats, cells were cultured in MEM-D-valine-S with 10% FBS-DCC for 3 d and then for 2 d in the same fresh medium containing 17β-estradiol (10^{-9} M) or vehicle (ethanol final concentration, 1 μl/liter). Finally, the cells were incubated for another day in MEM-D-valine-SS without FBS in the presence of 17β-estradiol and TNF-α (50 ng/ml).

Metabolic activity determination

The metabolic activity of viable cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were washed twice and incubated for 4 h in 100 μl Krebs buffer plus 50 μg MTT reagent dissolved in 10 μl PBS at 37 C. The developed crystals were dissolved in 100 μl 0.04 N HCl in isopropanol, and the OD was read in a microplate spectrophotometer at a wavelength of 600 nm.

Microscopic determination of DNA fragmentation by the TUNEL method

After the culture period, cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized by microwave irradiation (26). DNA strand breaks were labeled with digoxigenin-deoxy-UTP using terminal deoxynucleotidyl transferase (0.18 U/μl) according to manufacturer's protocol. The incorporation of nucleotides into the 3′-OH end of damaged DNA was detected with an antidigoxigenin-fluorescein antibody and was visualized with a fluorescence microscope.

Microscopic determination of apoptotic nuclear morphology

Cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized with 0.2% Triton X-100 (vol/vol) in PBS. The coverslides were preincubated with 1 mg/ml ribonuclease A for 30 min and then incubated with 1 μg/ml propidium iodide for 15 min. Cell death was analyzed by nuclear chromatin morphology. Apoptotic cells were identified by the presence of nuclear condensation and/or chromatin margination or fragmentation (26, 27). The percentage of apoptotic cells was defined as apoptotic cells × 100/total cells.

Immunofluorescent identification of anterior pituitary cells

Anterior pituitary cells were identified by indirect immunofluorescence staining. Cultured cells were fixed as described above and incubated with 5% normal rabbit serum in PBS with 0.2% Triton X-100 (vol/vol) for 30 min. Then slides were incubated with primary antibodies [guinea pig antirat PRL (NHPP-IC, 1:2500), guinea pig antirat GH (NHPP-IC, 1:2000), guinea pig antirat βLH (NHPP-IC-2, 1:5000), guinea pig antirat ACTH (NHPP-IC-1, 1:400)] in PBS containing 0.2% Triton X-100 (vol/vol) and 0.5% normal rabbit serum for 1 h. After rinsing, slides were incubated for 1 h with rabbit antiguinea pig fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies at a 1:200 dilution in the same buffer. Cells were observed under a fluorescent light microscope (Axiophot, Carl Zeiss, Jena, Germany) with mounting medium for fluorescence (Vectashield, Vector Laboratories, Inc., Burlingame, CA). Control slides were incubated with normal guinea pig serum instead of primary antibody.

Electrophoretic determination of DNA fragmentation

Isolation and separation of apoptosis-associated DNA fragments in anterior pituitary cells were performed according to the method described by Steinfelder *et al.* (28). Briefly, cells were rinsed twice with PBS and scraped off the wells. The cells were centrifuged at 500 × *g* for 5 min and lysed with 0.6% sodium dodecyl sulfate in Tris-EDTA (TE) buffer, pH 7.5, with 1 μg/μl ribonuclease A. After precipitation with 3 M CsCl, 1 M potassium acetate, and 0.67 M acetic acid, the mixture was spun at 14,000 × *g* for 15 min. The supernatant was spun in a MiniPrep spin column (QIAprep Spin Miniprep Kit, QIAGEN, Hilden, Germany) at 14,000 × *g* for 1 min, washed, and spun again. DNA was eluted with TE buffer, pH 8.0, run into 1.6% acetic acid/TE (pH 7.6) agarose gel, and stained with ethidium bromide.

Statistical analysis

Viability data were expressed as the mean ± SE and were evaluated by *t* test. Differences were considered significant if $P < 0.05$. The experiments were performed at least twice. Results from individual experiments are shown in the figures. The number of apoptotic cells (as identified by nuclear morphology of cells stained with propidium iodide or by the TUNEL method) was analyzed in duplicate slides for each cell preparation, and each group included at least two independent experiments. Results were expressed as the percentage of apoptotic cells of the total number of cells counted of each specific population or condition. Differences between proportions were analyzed by the χ^2 test with 95% confidence. In estrous cycle studies, the relative risk of occurrence of apoptosis after exposition to TNF-α was defined as the ratio of proportions of apoptosis in the presence and absence of TNF-α in cells from rats killed at each stage of the estrous cycle. Differences between relative risks were considered significant when 95% confidence intervals, calculated by the approximation of Katz, did not overlap (InStat version 3.00, GraphPad Software, Inc., San Diego, CA).

Results

Effect of TNF-α on the metabolic activity of anterior pituitary cells

To study the effect of TNF-α on anterior pituitary cell viability, we first evaluated its action on the metabolic ac-

tivity of these cells by MTT assay. TNF-α (50 ng/ml) decreased the metabolic activity of anterior pituitary cells from pooled intact rats within 6 h and for up to 48 h when cells were incubated in serum-free MEM-D-valine (Fig. 1). This inhibitory effect was abrogated when the cells were incubated for 24 h with TNF-α in the presence of 2.5% FBS (data not shown). Therefore, in the following experiments, the incubations with TNF-α were performed in serum-free MEM-D-valine culture medium for 24 h.

Effect of TNF-α on percentage of apoptotic anterior pituitary cells

The decrease in metabolic activity of viable cells induced by TNF-α could result from inhibition of cell proliferation or induction of cell death. We have already demonstrated that TNF-α inhibits cell proliferation (12). Therefore, we evaluated whether TNF-α also induces cell death of anterior pituitary cells from rats killed at random stages of the estrous cycle. TNF-α (50 ng/ml) significantly increased the percentage of TUNEL-positive cells (control, 5.38%; TNF-α, 7.69%; $P < 0.001$). Staining with propidium iodide permitted observation of the morphological features of apoptotic cells, such as nuclear condensation, chromatin fragmentation and margination, and the presence of apoptotic bodies (Fig 2).

To identify which of the cell populations of the anterior pituitary were committed to apoptosis by TNF-α, we determined the number of immunoreactive cells for PRL, GH, ACTH, and βLH with nuclear apoptotic morphology revealed by propidium iodide. TNF-α increased the percentage of somatotropes and lactotropes with apoptotic morphology, but did not significantly affect the proportion of apoptotic corticotropes or gonadotropes (Fig. 3). The induction of apoptosis in lactotropes by TNF-α was also detected by the TUNEL method (Fig. 4).

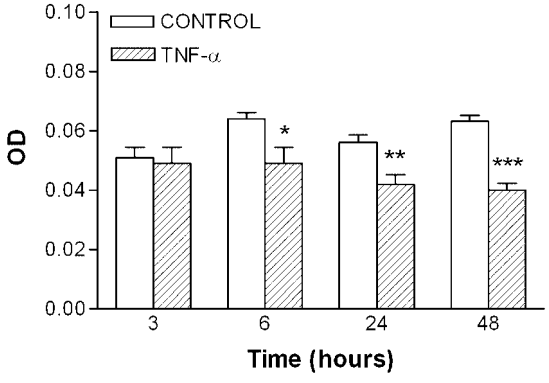

FIG. 1. Effect of TNF-α on the metabolic activity of viable anterior pituitary cells. Cells from rats killed at random stages of the estrous cycle were cultured in MEM-D-valine-S (10% FBS) for 3 d. Then, the medium was replaced by MEM-D-valine-SS without FBS, and the cells were cultured for another 3 d. TNF-α (50 ng/ml) was added at the time specified in the figure before termination of the culture. Cell viability was assessed by the MTT assay. Each *column* represents the mean ± SE of 10 wells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* respective control).

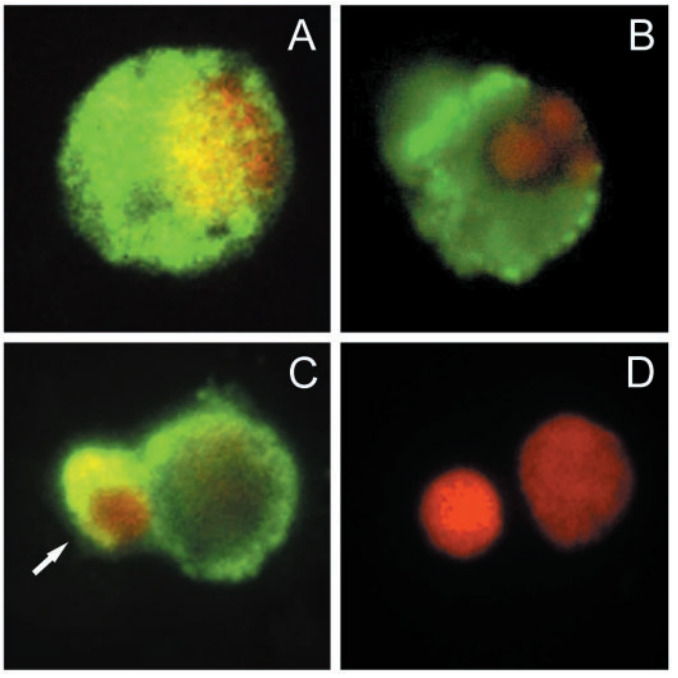

FIG. 2. Apoptotic lactotrope and somatotrope. Representative anterior pituitary cells showing immunocytochemistry for PRL or GH and counterstaining with propidium iodide (magnification, ×1500). A, Normal lactotrope. B, A PRL-immunopositive cell exhibits nuclear chromatin fragmentation indicative of apoptosis. C, Two GH-immunopositive cells. The *arrow* shows a shrunken cell with condensed nucleus. D, Same cells as in C, showing nuclear morphology.

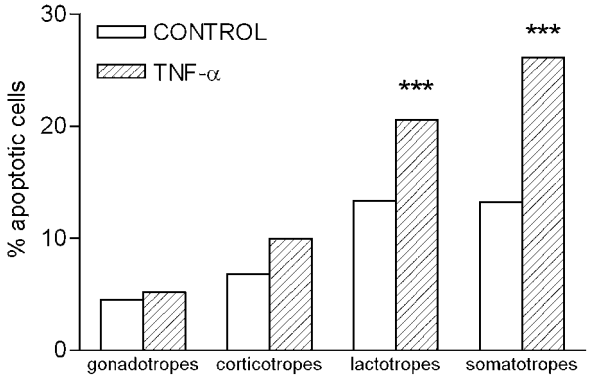

FIG. 3. Effect of TNF-α on the percentage of apoptotic cells in different cell populations of the anterior pituitary. Anterior pituitary cells from rats killed at random stages of the estrous cycle were cultured for 3 d in MEM-D-valine-S (10% FBS), for 1 d in MEM-D-valine-SS without FBS, and then for 1 d in the same fresh medium containing TNF-α (50 ng/ml). Each *column* represents the percentage of apoptotic cells (as identified by nuclear morphology of cells stained with propidium iodide) of the total number of cells counted of each specific population (n = 300-1800 cells from at least 4 separate experiments). ***, $P < 0.001$ (*vs.* respective control).

Apoptotic effect of TNF-α on anterior pituitary cells from rats killed at selected stages of the estrous cycle

As TNF-α release from anterior pituitary cells varies throughout the estrous cycle (12), we investigated the apoptotic effect of TNF-α on anterior pituitary cells from rats killed in proestrus, estrus, or diestrus. TNF-α significantly

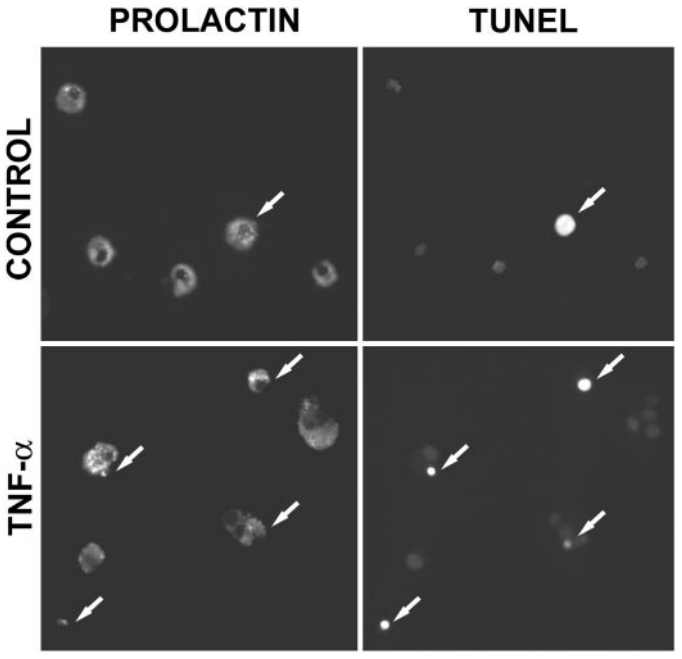

FIG. 4. TUNEL-positive lactotropes. Anterior pituitary cells from rats killed at random stages of the estrous cycle were cultured for 3 d in DMEM-S (10% FBS), for 1 d in DMEM-S without FBS, and then for 1 d in the same fresh medium containing TNF-α (50 ng/ml). Lactotropes were identified by immunocytochemistry (magnification, ×350).

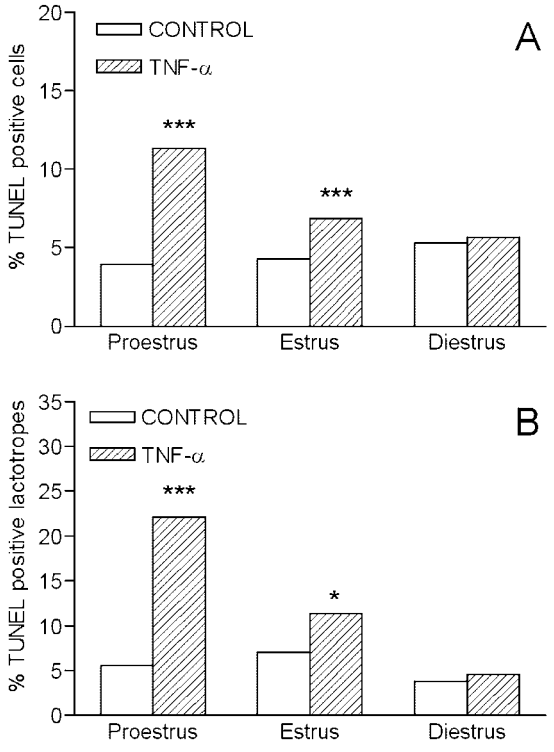

FIG. 5. Effect of TNF-α on the percentage of TUNEL-positive anterior pituitary cells (A) and lactotropes (B) of rats killed at selected stages of the estrous cycle. Cells from rats killed in proestrus, estrus, and diestrus were cultured for 2 d in MEM-D-valine-S (10% FBS-DCC) and then for 1 d in MEM-D-valine-SS without FBS containing TNF-α (50 ng/ml). Each *column* represents the percentage of TUNEL-positive cells of the total number of cells in each condition. A, Anterior pituitary cells (n > 2000 cells). B, Lactotropes (n > 400 cells). *, $P < 0.05$; ***, $P < 0.001$ (*vs.* respective control without TNF-α).

increased the percentage of TUNEL-positive cells from rats killed in proestrus and estrus, but not in diestrus (Fig. 5A). However, this effect was significantly greater ($P < 0.05$) in cells from rats killed in proestrus (relative risk, 2.88) than in estrus (relative risk, 1.60). Also, TNF-α significantly increased the percentage of apoptotic lactotropes in cultures of anterior pituitary cells from rats killed in proestrus and estrus; the apoptotic effect of TNF-α was significantly higher ($P < 0.05$) in cells from rats in proestrus (relative risk, 3.94) than in estrus (relative risk, 1.62; Fig. 5B).

In addition, agarose gel electrophoresis showed the typical laddering of apoptotic DNA fragmentation, indicating that TNF-α induced cell death by apoptosis. Breakdown of genomic DNA into high and low molecular weight fragments induced by TNF-α was clearly observed in anterior pituitary cells from rats killed in proestrus, but not in diestrus (Fig. 6).

Effect of estradiol on TNF-α-induced apoptosis

Considering that TNF-α promotion of apoptosis was predominant in anterior pituitary cells from rats killed in proestrus, we investigated whether TNF-α proapoptotic action is dependent on estrogens. For this purpose, anterior pituitary cells from OVX rats were cultured in the presence of 10^{-9} M 17β-estradiol. TNF-α significantly increased the percentage of anterior pituitary cells from OVX rats with apoptotic morphology only when cells were cultured in the presence of 17β-estradiol (Fig. 7A). A similar effect was observed in lactotropes from OVX rats (Fig. 7B).

Discussion

Our results show the presence of cells with apoptotic characteristics in all subpopulations of secretory cells of the anterior pituitary gland from intact female rats that we studied. Although the percentage of apoptotic anterior pituitary cells determined in basal culture conditions in serum-free medium was relatively low, it was markedly higher in some subpopulations, such as lactotropes and somatotropes. In these specific subpopulations, TNF-α induced death by apoptosis.

To date, receptors for TNF-α in the anterior pituitary have been found in two cell lines: AtT-20, deriving from corticotropes, and TtT/GF, deriving from folliculostellate cells (8). Although both receptor subtypes for TNF-α were identified in the mouse anterior pituitary gland, only TNFR-1 was detected in rat anterior pituitary. However, as TNFR-2 is a species-specific receptor, and its detection requires rat TNF-α, it is possible that both TNFR-1 and TNFR-2 are present in the rat anterior pituitary (9). Neither the presence of TNF receptors nor the relative expression of each subtype of TNFR in the different cell subpopulations of the anterior pituitary has been investigated, although they may account for the differences in the response of each cell subpopulation to the cytokine.

Death by apoptosis was reported in tumoral and normal

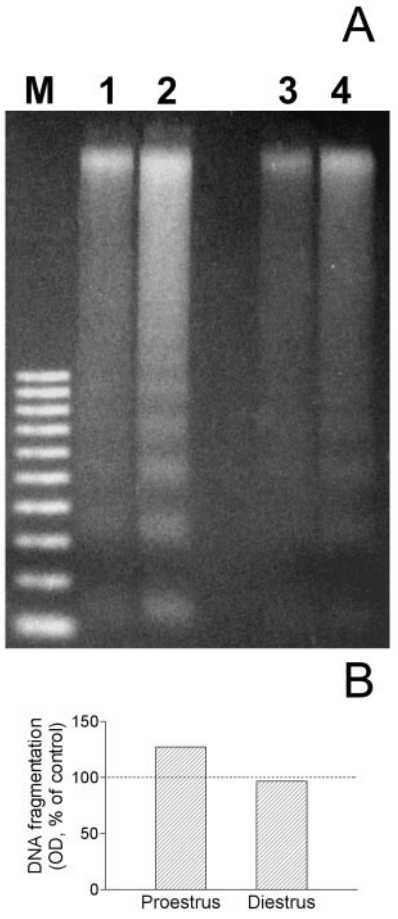

FIG. 6. TNF-α-induced DNA fragmentation. A, Anterior pituitary cells from rats killed in proestrus (lanes 1 and 2) and diestrus (lanes 3 and 4) were cultured for 2 d in MEM-D-valine-S (10% FBS-DCC) and then for 1 d in MEM-D-valine-SS without (lanes 1 and 3) or with TNF-α (50 ng/ml; lanes 2 and 4). DNA was isolated and analyzed by agarose gel electrophoresis as described in *Materials and Methods*. One representative gel of two experiments is shown. B, The digitalized gel image was analyzed using standard densitometric scanning software. The amount of fragmented DNA (200–1500 bp) was determined as the OD and expressed as a percentage of the respective control without TNF-α.

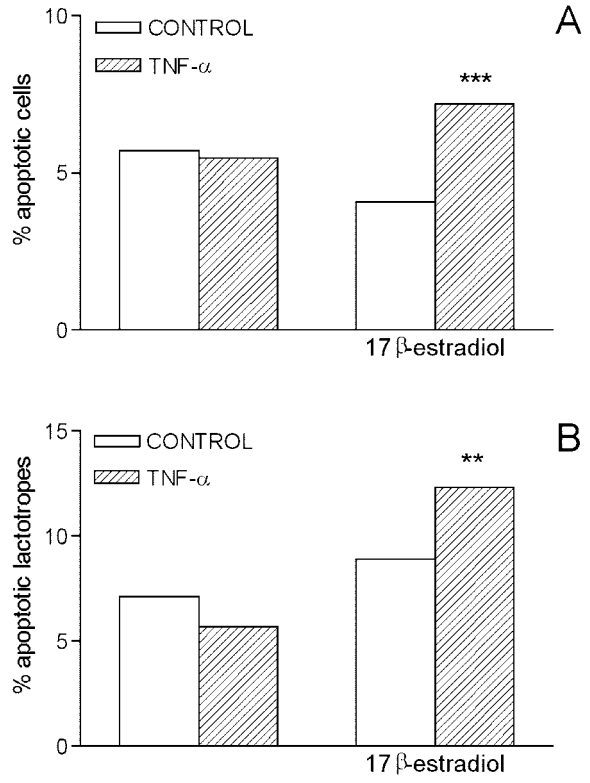

FIG. 7. Effect of TNF-α on the percentages of apoptotic anterior pituitary cells (A) and lactotropes (B) from OVX rats cultured in the presence of 17β-estradiol. Cells were cultured for 3 d in MEM-D-valine-S (10% FBS-DCC) and for 2 d in the same medium containing 17β-estradiol (10^{-9} M) or vehicle (ethanol 1 μl/liter). Finally, cells were incubated for another day in MEM-D-valine-SS without FBS in the presence of estradiol and TNF-α (50 ng/ml). Each *column* represents the percentage of apoptotic cells (as identified by nuclear morphology of cells stained with propidium iodide). A, Anterior pituitary cells (n > 4200). B, Lactotropes (n > 1000). **, $P < 0.01$; ***, $P < 0.001$ (*vs.* respective control without TNF-α).

anterior pituitary cells (19–21, 29). Treatment of adrenalectomized rats with dexamethasone was shown to induce a burst of apoptosis of corticotropes in the anterior pituitary with concurrent suppression of mitosis (30). Tissue remodeling occurring after termination of lactation involves apoptosis (22). Apoptosis was also reported to be induced by bromocriptine in anterior pituitary glands from estrogen-stimulated female rats (31).

Our results indicate that TNF-α exerts a proapoptotic effect on anterior pituitary cells that varies throughout the estrous cycle and is predominant in cells of proestrus rats. Although it cannot be assured that cells from rats killed at a specific stage of the estrous cycle preserve their original characteristics in culture, the differential apoptotic response to TNF-α according to the stage in which the animals were killed suggest the cyclicity of such response. A higher apoptotic rate was also induced by TNF-α in lactotropes of anterior pituitaries from rats killed in proestrus, suggesting that the proapoptotic action of this cytokine is dependent on gonadal steroids. In fact, TNF-α increased the apoptosis of total anterior pituitary cells, specifically of lactotropes from OVX rats, only when these cells were cultured in the presence of 17β-estradiol.

It has been demonstrated that inhibitors of apoptosis proteins, such as the X-linked inhibitor of apoptosis protein, whose expression is regulated by TNF-α via nuclear factor-κB, modulate TNF-α death signaling pathways in granulosa cells (32). Thus, the inability of lactotropes to undergo apoptosis after TNF-α challenge in the absence of estrogens could be due in part to an imbalance in the expression of proapoptotic and antiapoptotic factors. Breaking with the current dogma that sex steroids are required for the growth and development of hormone-sensitive cancer, steroid hormones have been suggested recently to be mediators of both cell proliferation and cell death (33). Dihydrotestosterone enhances transforming growth factor-β-induced apoptosis in a hormone-sensitive prostate cancer cell line through mechanisms involving apoptotic regulatory proteins such as Bcl-2 (34). Further, high concentrations of estradiol increase apoptosis of cell lines derived from breast cancer with long-term deprivation of estrogens, probably by activation of a Fas-

mediated mechanism (35). Also, some effects of estradiol, *i.e.* neuroprotection, rely upon the expression of estrogen receptor α (ERα), whereas expression of ERβ without ERα potentiates apoptosis (36). Therefore, it was suggested that divergent effects of estradiol may depend on which ER subtype is expressed. ERα may up-regulate antiapoptotic genes, whereas when there is a cell death signal, activated ERβ could potentiate this signal (36). Lactotropes are accepted to be a major target for estrogens that stimulate PRL synthesis and release as well as lactotrope proliferation (37). ERα and ERβ isoforms have been detected in gonadotropes, corticotropes, lactotropes, and folliculostellate cells. Only a small percentage of anterior pituitary cells coexpress both ERα and ERβ. Approximately 50% of the lactotropes express ERα, whereas less than 30% express ERβ (38). Nevertheless, ERβ mRNA was found to be relatively more abundant in cells with lactotrope characteristics (39). Lactotropes also have the highest levels of the pituitary-specific truncated ER product-1 (TERP-1). Its expression and the expression of ERα and ERβ in the pituitary may be regulated by gonadal steroids during the estrous cycle. The expression of TERP-1 shows a dramatic increase from the morning of diestrus to the afternoon of proestrus, whereas ERβ mRNA levels fall at proestrus, presumably induced by rising estradiol levels (38, 39). Lactotrope cell proliferation varies in different physiological conditions, such as pregnancy, lactation, and the estrous cycle (40, 41). During the estrous cycle, lactotrope proliferation was observed only during estrus and not at any other stage (41), and it has been suggested that lactotrope proliferation requires both stimulation of secretion of hypothalamic factors and the sensitizing action of estradiol (42). Homeostatic control of cell number results from a dynamic balance between the number of cells generated and the number of cells undergoing apoptotic death. We previously reported that TNF-α inhibition of anterior pituitary cell proliferation was estrogen dependent (12). Our present data indicate that TNF-α-induced apoptosis of lactotropes also requires the presence of estrogens. This estrogen dependency of both inhibition of proliferation and induction of apoptosis may result in a reduction of the lactotrope population at a specific stage of the estrous cycle, providing one means for terminating the proestrus PRL surge. We propose that the lactotrope population is renewed in each estrous cycle by cell proliferation during estrus and cell death by apoptosis during proestrus. Both events may respond to gonadal steroids, probably depending on the relative expressions of different ER isoforms. In fact, one hypothesis is that TERP-1 suppresses estrogen action in the pituitary at proestrus, intervening in the termination of the proestrus surge (39). As TNF-α release from anterior pituitary cells is higher during proestrus (12), our results suggest that this cytokine may participate as an autocrine/paracrine factor in anterior pituitary cell renewal during the estrous cycle.

In cycling rats, PRL and gonadotropin surges occur during the afternoon of proestrus, whereas the serum levels of these hormones remain low at other stages of the estrous cycle. Serum GH levels appear to be unrelated to the rat estrous cycle (43). However, a rise of serum GH was observed near the peak of the gonadotropin surge in ewes (44). Moreover, the expression of GH mRNA reaches a peak in proestrus rats (45). Evidence indicates that pituitary acidophils are a dynamic cell population capable of functional interconversion. The differentiation of lactotropes from somatotropes includes a transitional cell type, the somatomammotropes, that express ERs (46, 47). A subpopulation of somatotropes also expresses LH and FSH mRNAs (48). In our study, TNF-α induced apoptosis of somatotropes, an event that could restrain somatotrope expansion. Although we did not dual-label secretory cells, it is possible that part of the somatotrope population that died by apoptosis could be dual-hormone secretors or multihormonal cells. Even though lactotropes account for almost all the mitotic activity detected in the anterior pituitary during the estrous cycle (41), recruitment and maturation of gonadotropes occur during diestrus in cycling female rats. Several factors, such as estradiol (46), epidermal growth factor, and GnRH (49), are known to be mitogens for gonadotropes. Therefore, it is possible to speculate that a number of gonadotropes may die to balance the new cells generated in each estrous cycle. TNF-α did not modify the percentage of apoptotic gonadotropes. However, paracrine factors other than TNF-α could be involved in the control of gonadotrope population homeostasis.

In conclusion, our results indicate that TNF-α induces apoptosis of lactotropes by an estrogen-dependent mechanism. This apoptotic effect may be predominant at proestrus and could participate in the homeostatic maintenance of cell number in the anterior pituitary. As the mechanisms involved in apoptosis are tightly regulated, alterations in their function could be involved in the pathogenesis of pituitary tumors.

Acknowledgments

Received April 4, 2002. Accepted May 29, 2002.

Address all correspondence and requests for reprints to: Dr. Adriana Seilicovich, Centro de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 10, Buenos Aires 1121, Argentina. E-mail: adyseili@fmed.uba.ar.

This work was supported by grants from Agencia Nacional de Investigaciones Científicas y Tecnológicas, Ministerio de Salud, CONICET, and Universidad de Buenos Aires, Argentina.

References

1. **Semenzato G** 1990 Tumor necrosis factor: a cytokine with multiple biological activities. Br J Cancer 61:354–361
2. **Leong KG, Karsan A** 2000 Signaling pathways mediated by tumor necrosis factor α. Histol Histopathol 15:1303–1325
3. **Jansky L, Vybíral S, Ppopísilová D, Roth J, Dornand J, Zeisberger E, Kamínková J** 1995 Production of systemic and hypothalamic cytokines during the early phase of endotoxic fever. Neuroendocrinology 62:55–61
4. **Nadeau S, Rivest S** 1999 Regulation of the gene encoding tumor necrosis factor α (TNF-α) in rat brain and pituitary in response in different models of systemic immune challenge. J Neuropathol Exp Neurol 58:61–77
5. **Turnbull AV, Rivier CL** 1999 Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanism of action. Physiol Rev 79:1–71
6. **Theas MS, De Laurentiis A, Lasaga M, Pisera D, Duvilanski B, Seilicovich A** 1998 Effect of lipopolysaccharide on tumor necrosis factor and prolactin secretion from rat anterior pituitary cells. Endocrine 8:241–245
7. **Arras M, Höche A, Bohle R, Eckert P, Riedel W, Schaper J** 1996 Tumor necrosis factor-α in macrophages of heart, liver, kidney, and in the pituitary gland. Cell Tissue Res 285:39–49
8. **Kobayashi H, Fukata J, Murakami N, Usui T, Ebisui O, Muro S, Hanaoka I, Inoue K, Imura H, Nakao K** 1997 Tumor necrosis factor receptor in the pituitary cells. Brain Res 758:45–50
9. **Wolvers DAW, Marquette C, Berkenbosch F, Haour F** 1993 Tumor necrosis

factor-α binding sites in rodent brain and pituitary gland. Eur Cytokine Network 4:377–381
10. **Ray D, Melmed S** 1997 Pituitary cytokine and growth factor expression and action. Endocr Rev 18:206–228
11. **Artz E, Páe Zpereda M, Pere Zcastro C, Pagotto U, Renner U, Stalla GK** 1999 Pathophysiological role of the cytokine network in the anterior pituitary. Front Neuroendocrinol 20:71–95
12. **Theas S, Pisera D, Duvilanski B, De Laurentiis A, Pampillo M, Lasaga M, Seilicovich A** 2000 Estrogens modulate the inhibitory effect of tumor necrosis factor-α on anterior pituitary growth and prolactin release. Endocrine 12: 249–255
13. **Ksontini R, MacKay SLD, Moldawer LL** 1998 Revisiting the role of tumor necrosis factor α and the response to surgical injury and inflammation. Arch Surg 133:558–567
14. **Arch RH, Gedrich RW, Thompson CB** 1998 Tumor necrosis factor receptor-associated factors (TRAFs): a family of adapter proteins that regulates life and death. Genes Dev 12:2821–2830
15. **Thornberry NA, Lazebnik Y** 1998 Caspases: enemies within. Science 281: 1312–1316
16. **Green DR, Reed JC** 1998 Mitochondria and apoptosis. Science 281:1309–1312
17. **Dopp JM, Mackenzie A, Otero GC, Merril JE** 1998 Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. J Neuroimmunol 75:104–112
18. **Ashkenazi A, Dixit VM** 1998 Death receptors: signaling and modulation. Science 281:1305–1308
19. **Nolan LA, Kavanagh E, Lightman SL, Levy A** 1998 Anterior pituitary cell population control: basal cell turnover and the effects of adrenalectomy and dexamethasone treatment. J Neuroendocrinol 10:207–215
20. **Drewett N, Jacobi JM, Willgoss DA, Lloyd HM** 1993 Apoptosis in the anterior pituitary gland of the rat: studies with estrogen and bromocriptine. Neuroendocrinology 57:89–95
21. **Green VL, White MC, Hipking LJ, Jeffreys RV, Foy PM, Atkin SL** 1997 Apoptosis and p53 suppressor gene protein expression in human anterior pituitary adenomas. Eur J Endocrinol 136:382–387
22. **Ahlbom EA, Grandison L, Zhivotovsky B, Ceccatelli S** 1998 Termination of lactation induces apoptosis and alters the expression of the Bcl-2 family members in the rat anterior pituitary. Endocrinology 139:2465–2471
23. **Thompson EB** 1994 Apoptosis and steroid hormones. Mol Endocrinol 8: 665–673
24. **De M, Sandford TR, Wood GW** 1992 Interleukin-1, interleukin-6, and tumor necrosis factor-α are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. Dev Biol 151:297–305
25. **Roby KF, Hunt JS** 1994 Mouse endometrial tumor necrosis factor-α messenger ribonucleic acid and protein: localization and regulation by estradiol and progesterone. Endocrinology 135:2780–2789
26. **Negoescu A, Lorimier P, Labat-Moleur F, Drout C, Robert C, Guillermet C, Brambilla C, Brambilla E** 1996 In situ apoptotic cell labeling by The TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 9:959–968
27. **Huppertz B, Frank H-G, Kaufman P** 1999 The apoptosis cascade-morphological and immunohistochemical methods for its visualization. Anat Embryol 200:1–18
28. **Steinfelder HJ, Quentín I, Ritz V** 2000 A fast and sensitive technique to study the kinetics and the concentration dependencies of DNA fragmentation during drug-induced apoptosis. J Pharmacol Toxicol Methods 43:79–84
29. **Vidal S, Horvath E, Kovacs K, Scheithauer BW, Lloyd RV, Kontogeorgos G** 2001 Ultrastructural features of apoptosis in human pituitary adenomas. Ultrastruct Pathol 25:85–92
30. **Nolan LA, Levy A** 2001 Anterior pituitary trophic responses to dexamethasone withdrawal and repeated dexamethasone exposures. J Endocrinol 169:263–270
31. **Yonezawa K, Tamaki N, Kokunai T** 1997 Effects of bromocriptine and terguride on cell proliferation and apoptosis in the estrogen-stimulated anterior pituitary gland of the rat. Neurol Med Chir 37:901–906
32. **Xiao CW, Ash K, Tsang BK** 2001 Nuclear factor-κB mediated X-linked inhibitor of apoptosis protein expression prevents rat granulosa cells from tumor necrosis factor α-induced apoptosis. Endocrinology 142:557–563
33. **Soto AM, Sonnenschein C** 2001 The two faces of janus: sex steroids as mediators of both cell proliferation and cell death. J Natl Cancer Inst 93:1673–1675
34. **Bruckheimer EM, Kyprianou N** 2001 Dihydrotestosterone enhances transforming growth factor-β-induced apoptosis in hormone-sensitive prostate cancer cells. Endocrinology 142:2419–2426
35. **Song RX, Naftolin MG, McPherson RA, Song J, Zhang Z, Yu W, Wang J, Santen RJ** 2001 Effect of long-term deprivation on apoptotic responses of breast cancer cells to 17β-estradiol. J Natl Cancer Inst 93:1714–1723
36. **Nilsen J, Mor G, Naftolin F** 2000 Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system. J Neurobiol 43:64–78
37. **Allen DL, Mitchner NA, Uveges TE, Nephew KP, Khan S, Ben-Jonathan N** 1997 Cell-specific induction of c-fos expression in the pituitary gland by estrogen. Endocrinology 138:2128–2135
38. **Mitchner NA, Garlick C Ben-Jonathan N** 1998 Cellular distribution and gene regulation of estrogen receptors α and β in the rat pituitary gland. Endocrinology 139:3976–3983
39. **Schreihofer DA, Stoler MH, Shupnik MA** 2000 Differential expression and regulation of estrogen receptors (ERs) in rat pituitary and cell lines: estrogen decreases ERα protein and estrogen responsiveness. Endocrinology 141:2174–2184
40. **Oishi Y, Okuda M, Takahashi H, Fujii T, Morii S** 1993 Cellular proliferation in the anterior pituitary gland of normal adult rats: influences of sex, estrous cycle, and circadian change. Anat Rec 235:111–120
41. **Hashi A, Mazawa S, Kato J, Arita J** 1995 Pentobarbital anesthesia during the proestrus afternoon blocks lactotroph proliferation occurring on estrus in female rats. Endocrinology 136:4665–4671
42. **Yin P, Arita J** 2000 Differential regulation of prolactin release and lactotrope proliferation during pregnancy, lactation and the estrous cycle. Neuroendocrinology 72:72–79
43. **Shimokawa N, Kato Y, Imai K, Wakabayashi K** 1990 Changes in content of prolactin mRNA during the rat estrous cycle. Exp Clin Endocrinol 96:8–14
44. **Ladenfeld TD, Suttie JM** 1989 Changes in messenger ribonucleic acid concentrations and plasma levels of growth hormone during the ovine estrous cycle and in response to exogenous estradiol. Endocrinology 125:1474–1478
45. **Childs GV, Unabia G, Wu P** 2000 Differential expression of growth hormone messenger ribonucleic acid by somatotropes and gonadotropes in male and female cycling rats. Endocrinology 141:1560–1570
46. **Asa Sl, Ezzat S** 1998 The cytogenesis and pathogenesis of pituitary adenomas. Endocr Rev 19:798–827
47. **Kineman RD, Henricks DM, Faught WJ, Frawley LS** 1991 Fluctuations in the proportions of growth hormone and prolactin-secreting cells during the bovine estrous cycle. Endocrinology 129:1221–1225
48. **Childs GV, Unabia G, Miller BT, Collins TJ** 1999 Differential expression of gonadotropin and prolactin antigens by GHRH target cells from male and female rats. J Endocrinol 162:177–187
49. **Childs GV, Unabia G** 2001 Epidermal growth factor and gonadotropin-releasing hormone stimulate proliferation of enriched population of gonadotropes. Endocrinology 142:847–853