Adenoviral vectors encoding tumor necrosis factor- α and FasL induce apoptosis of normal and tumoral anterior pituitary cells

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Abstract

Our previous work showed that tumor necrosis factor (TNF)- α and FasL induce apoptosis of anterior pituitary cells. To further analyze the effect of these proapoptotic factors, we infected primary cultures from rat anterior pituitary, GH3 and AtT20 cells with first-generation adenoviral vectors encoding TNF- α , FasL or, as a control, β -galactosidase (β -Gal), under the control of the human cytomegalovirus promoter. Successful expression of the encoded transgenes was determined by immunocytochemistry. Although we observed basal expression of TNF- α and FasL in control cultures of anterior pituitary cells, fluorescence-activated cell sorting (FACS) cell cycle analysis showed that the overexpression of TNF- α or FasL increases the percentage of hypodiploid lactotrope and somatotrope cells. Nuclear morphology and TUNEL staining revealed that the cells undergo an apoptotic death process. We detected strong immunoreactivity for TNFR1 and

Fas in the somatolactotrope cell line GH3. TNF- α , but not FasL, was expressed in control cultures of GH3 cells. The infection of GH3 cells with adenovirus encoding TNF- α or FasL increased the percentages of hypodiploid and TUNEL-positive cells. TNF- α or FasL immunoreactivity was not observed in the corticotrope cell line AtT20. However, adenovirus encoding TNF- α or FasL efficiently transduced these cells and increased the percentages of hypodiploid and TUNEL-positive cells. The expression of β -Gal was detected in all these cultures but did not affect cell viability. In conclusion, these results suggest that death signaling cascades triggered by TNF receptor 1 (TNFR1) and Fas are present in both normal and tumoral pituitary cells. Therefore, overexpression of proapoptotic factors could be a useful tool in the therapy of pituitary adenomas.

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Introduction

Although most pituitary tumors are benign, aggressive local growth can occur. Altered pituitary hormone release often leads to serious clinical disorders, such as acromegaly, Cushing's disease and sexual dysfunction (Melmed 2003). Pituitary tumors arise from any of the highly differentiated cell types present in this gland. The genesis of pituitary adenomas is believed to involve cell transformation due to genetic mutations, followed by central, peripheral and local signals that induce cell proliferation, thus stimulating tumor progression (Asa & Ezzat 2002). Supporting the theory of intrinsic pituitary defects is the monoclonal nature of pituitary adenomas (Alexander *et al.* 1990, Herman *et al.* 1990). Considering that the regulation of tissue homeostasis results from the balance between cell proliferation and death, and that the cells bearing genetic alterations are physiologically elimi-

nated by apoptosis, defects in cell death processes play an important role in tumor genesis and progression (Norbury & Zhitovitsky 2004). Also, evasion of cell death can lead to the selection of immortal transformed cells (Eischen *et al.* 2001).

Death receptors, such as Fas (receptor for FasL), tumor necrosis factor receptor 1 (TNFR1; one of the receptors for TNF) and DR5 (receptor for TNF-related apoptosis-inducing ligand (TRAIL)) are type-I membrane proteins that have a conserved domain called the death domain. Upon binding to the specific ligand, death receptors form trimers (Ashkenazi & Dixit 1998, Evan & Littlewood 1998). The apoptotic signal transduction cascade that is induced by death receptor activation has been well characterized. Ligation of Fas by FasL or TNFR1 by TNF- α results in formation of a death-inducing signalling complex (DISC), which initiates a cascade of events that, through activation of caspases 8 and 10, leads to apoptosis

(Scaffidi *et al.* 1999, Chen & Goeddel 2002). The initiator caspases can either directly activate executioner caspase 3 or cleave Bid, a proapoptotic member of the Bcl-2 family. The truncated Bid translocates to the mitochondria stimulating cytochrome *c* release (Gonzalez *et al.* 2005). Cytochrome *c*, together with Apaf-1 activates caspase 9, which in turn activates caspase 3 (Cecconi 1999). Then, caspase 3 and other downstream caspases cleave substrates involved in cell disassembling (Degeretev *et al.* 2003).

The anterior pituitary gland undergoes a process of cell renewal during the estrous cycle in the female rat (Hashi *et al.* 1995, Yin & Arita 2002). We have previously reported that TNFR1 and Fas activation induce apoptosis of anterior pituitary cells from female rats (Candolfi *et al.* 2002, 2004, 2005, Jaita *et al.* 2005b) suggesting that TNF- α and FasL are involved in the maintenance of tissue homeostasis in the anterior pituitary gland and could have potential benefits for the treatment of pituitary diseases. Although major advances have been made in the therapy of pituitary tumors, only partial success has been possible and novel therapies are needed. Thus, we explored the effect of first-generation recombinant adenoviral vectors (RAd) encoding TNF- α and FasL in normal and tumoral anterior pituitary cells. Our previous results have demonstrated the persistent high efficiency of transgene expression of adenoviral vectors encoding β -galactosidase or herpes simplex virus (HSV) Type 1 thymidine kinase in the anterior pituitary gland (Castro *et al.* 1997, Southgate *et al.* 2000b, 2001, Smith-Arica *et al.* 2001). In the present work, we tested the ability of adenoviral vectors encoding TNF- α (RAd-hCMV-TNF- α) or FasL (RAd-hCMV-FasL) to infect and express these proapoptotic transgenes within anterior pituitary cells from two different rat strains and also in the pituitary cell lines GH3 and AtT20. We investigated the proapoptotic effect of the vectors expressing TNF- α and FasL by fluorescence-activated cell sorting (FACS) analysis and the TUNEL method in both normal and tumoral pituitary cells. We found that adenoviral vectors encoding these proapoptotic genes are successfully expressed in pituitary cells, triggering the apoptotic cascade. Our results suggest that death receptors could possibly be considered as a target for the treatment of pituitary adenomas.

Material and Methods

Drugs

All culture media and supplements were obtained from Invitrogen, except fetal bovine serum (Biomed, Foster City, CA, USA) and the materials indicated below. All TUNEL reagents were purchased from Roche. The primary antibodies against anterior pituitary hormones were purchased from Dr A Parlow at the National Hormone and Pituitary Program (Torrance, CA, USA). Secondary antibodies were obtained from DAKO

(Glostrup, Denmark) and Chemicon International (Temecula, CA, USA). Avidin and biotin blocking solutions, fluorescein-avidin, diaminobenzidine (DAB), Vectastain ABC Elite kit and Vectashield were from Vector Laboratories Inc (Burlingame, CA, USA).

Adenoviral vectors

The RAd vectors used are based on adenovirus type 5 (Ad5), in which the left end of E1 and a portion of the E3 regions are deleted (E1 - /E3 -), and a cassette containing a recombinant exogenous gene and promoter is inserted in place of the E1 deletion. Three different vectors were used: RAd-hCMV-TNF- α (Ehtesham *et al.* 2002), RAd-hCMV-FasL (Larregina *et al.* 1998, Morelli *et al.* 1999, Maleniak *et al.* 2001) and RAd-hCMV- β -Gal (Castro *et al.* 1997). The RAds were grown and purified as previously described (Lowenstein *et al.* 1996, Southgate *et al.* 2000a).

Animals

Adult female Wistar and Sprague-Dawley rats were kept in controlled conditions of light (12 h light: 12 h darkness cycles) and temperature (20–25 °C). Standard laboratory chow and water were freely available and rats were kept in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Anterior pituitary glands were removed within minutes after rats were killed by breathing CO₂, followed by physical induction of pneumothorax.

Cell culture

A pool of anterior pituitary cells from five rats was used for each primary cell culture. Anterior pituitary glands were washed several times with Dulbecco's modified Eagle's medium (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 DNase (deoxyribonuclease II, Type V from bovine spleen) and 1 mg/ml trypsin inhibitor (Type II-S from soybean), and finally dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca²⁺ and Mg²⁺. Dispersed cells were washed twice and resuspended in DMEM supplemented with 10 μ l/ml MEM aminoacids, 2 mM glutamine, 5.6 μ g/ml amphotericin B and 25 μ g/ml gentamicin (DMEM-S or MEM-D-valine-S). Cell viability, as assessed by trypan blue exclusion, was over 90%. Cells were cultured in DMEM-S with 10% heat-inactivated fetal bovine serum for 1–2 days (37 °C, 5% CO₂ in air) before RAd virus infection.

GH3 and AtT20 cells (Castro *et al.* 1997) were cultured in MEM containing 10 μ l/ml non-essential aminoacids,

10 μ l/ml L-glutamine, 10 μ l/ml penicillin/streptomycin and 10% fetal calf serum 24 h prior to RAd virus infection.

Anterior pituitary cells, as well as GH3 and AtT20 cells were seeded onto coverslips in 24-well tissue culture plates (10×10^4 cells/0.5 ml per well) for the TUNEL method or immunocytochemistry, and onto 24-well tissue culture plates (25×10^4 cells/0.5 ml per well) for flow cytometry (FACS). Then, cells were infected for 48 h with different doses of viral vectors expressed as infectious units/cell (IU/cell) of adenoviral vectors expressing human TNF- α (RAd-hCMV-TNF), murine FasL (RAd-hCMV-FasL) or the *Escherichia coli* enzyme, β -galactosidase (RAd-hCMV- β -Gal) under the control of the human cytomegalovirus (hCMV) promoter (Southgate *et al.* 2000b, Windeatt *et al.* 2000). After this period, cells were fixed for immunocytochemistry and TUNEL or flow cytometric analysis.

Immunocytochemistry of TNFR1, TNF- α , Fas, FasL and β -Gal

We identified the presence of TNFR1, TNF- α , Fas, FasL and β -Gal in anterior pituitary cells by immunocytochemistry using rabbit anti-TNFR1 antibody (1:50; Stressgen, CA, USA), mouse anti-Fas antibody (1:50; Stressgen), rabbit anti-TNF- α (1:50; Endogen, IL, USA), rabbit anti-FasL antibody (1:25; Santa Cruz Biotech, CA, USA), and rabbit anti- β -Gal (1:1000, developed in our laboratory (Southgate *et al.* 2000b)) respectively. Briefly, after the culture period, cells were fixed with 4% formaldehyde in PBS for 30 min followed by an incubation with 10% normal donkey serum in PBS with 0.2% Triton X-100. After incubation with 0.3% H₂O₂ to inactivate endogenous peroxidase, or with avidin and biotin blocking solutions, cells were incubated overnight with primary antibodies in PBS containing 1% donkey serum. To detect β -Gal, cells were incubated with the primary antibody for 1 h. After rinsing, slides were incubated for 1 h with the corresponding biotinylated donkey anti-mouse or anti-rabbit IgG at a 1:200 dilution in the same buffer. Biotinylated antibodies were labeled with Vectastain ABC Elite kit, followed by staining with DAB and glucose oxidase (Sigma), and dehydration through graded ethanol solutions or with 2 μ g/ml fluorescein-conjugated avidin in 10 mM HEPES buffer, pH 7.5. Finally, slides were mounted with Vectashield, a mounting medium for fluorescence, containing DAPI (4',6-diamino-2-phenylindole dihydrochloride; Sigma) for DNA staining; slides were visualized in a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany) or a confocal microscope (Leica, Germany) or mounted with DPX-mounting medium (Fluka, Switzerland) for light microscopy. Control slides were incubated with the corresponding IgG subtype instead of primary antibody.

Microscopic determination of DNA fragmentation by the TUNEL method

The TUNEL method was performed as previously described (Candolfi *et al.* 2002). Briefly, after the culture period, cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized by microwave irradiation. DNA strand breaks were labeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase (0.18 U/ μ l) according to the manufacturer's protocol. The incorporation of nucleotides into the 3'-OH end of damaged DNA was detected with an anti-digoxigenin-fluorescein antibody.

Flow cytometric analysis (FACS)

Cultured cells were harvested with trypsin-EDTA, fixed with 75% ice-cold ethanol and permeabilized with 0.1% Triton-X100. Afterwards, cells were incubated in the presence of propidium iodide (50 μ g/ml) and RNase (10 μ g/ml) in PBS with 0.1% sodium azide. In the case of normal anterior pituitary cells, prior to incubation with propidium iodide, cells were incubated for 1 h with anti-rat prolactin (NHPP-IC, 1:1000) or anti-rat growth hormone (NHPP-IC, 1:2000) guinea pig primary antibodies in PBS containing 1% donkey serum and 0.1% sodium azide, followed by an incubation for 1 h with fluorescein-conjugated donkey anti-guinea pig secondary antibody. The percentage of hypodiploid cells (Sub-G1) was determined using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Percentages of hypodiploid (Sub-G1) cells, as analyzed by FACS, were expressed as means $\pm$ S.E. and evaluated by ANOVA followed by Dunnett's multiple comparison test. The number of apoptotic cells, as identified by the TUNEL method, was analyzed in duplicate slides from at least two independent experiments. Results were expressed as the percentage of apoptotic cells $\pm$ 95% confidence limits (CL) of the total number of cells counted in each specific condition. Confidence intervals for proportions were analyzed by the χ^2 test. $P < 0.05$ was used as the cut-off point for significance. All experiments were performed at least twice.

Results

TNF- α and FasL transgene expression in normal anterior pituitary cells

Our previous work showed that both TNF- α and FasL induce apoptosis of anterior pituitary cells (Candolfi *et al.* 2002, 2004, 2005, Jaita *et al.* 2005b). Now, we over-expressed TNF- α and FasL in primary cultures of anterior

pituitary cells by infecting them with first-generation adenoviral vectors encoding TNF- α (RAD-hCMV-TNF- α), FasL (RAD-hCMV-FasL) or, as a control, β -Gal (RAD-hCMV- β -Gal), under the control of the human cytomegalovirus promoter (hCMV). This promoter was previously shown to successfully drive expression of β -Gal in anterior pituitary cells and pituitary cell lines as well (Castro *et al.* 1997). All the encoded transgenes were efficiently transduced in infected anterior pituitary cells as detected by immunocytochemistry (Fig. 1).

In order to determine whether the overexpression of TNF- α or FasL induces apoptosis of anterior pituitary cells, we determined the percentage of hypodiploid cells in cultures of anterior pituitary cells from female Wistar rats after infection with increasing doses of RAD-hCMV-TNF- α , RAD-hCMV-FasL or RAD-hCMV- β -Gal. FACS cell cycle analysis showed that the infection of anterior pituitary cells with RAD-hCMV-TNF- α and RAD-hCMV-FasL increases the percentage of hypodiploid cells in a dose-dependent manner (Fig. 2A). In contrast, the expression of β -Gal had no cytotoxic effects in these cultures, even at the highest dose tested (Fig. 2A). Microscopic analysis of the nuclear morphology of RAD-hCMV-TNF- α - or RAD-hCMV-FasL-infected cultures of anterior pituitary cells revealed that cells exhibited characteristic features of an apoptotic death process, such as TUNEL staining in condensed and/or fragmented nuclei (data not shown). FACS cell cycle analysis of specific subpopulations of anterior pituitary cells infected with increasing concentrations of RAD-hCMV-TNF- α , RAD-hCMV-FasL or RAD-hCMV- β -Gal showed that adenoviral-driven expression of TNF- α or FasL exerts a dose-dependent propapoptotic effect in lactotropes (Fig. 2B). The infection with 50 infectious units/cell (IU/cell) of RAD-hCMV-FasL induced apoptosis of somatotropes, whereas a higher dose (100 IU/cell) of RAD-hCMV-TNF- α was required to induce an apoptotic effect in this cell subpopulation (Fig. 2C).

The propapoptotic effect of RAD-hCMV-TNF- α and RAD-hCMV-FasL was also observed in anterior pituitary cells from female Sprague-Dawley rats infected with 50 IU/cell of RAD-hCMV-TNF- α and RAD-hCMV-FasL. TNF- α and FasL overexpression increased the percentage

Figure 1 Expression of TNF- α (A) and FasL (B and C) in primary cultures of anterior pituitary cells infected with adenoviral vectors encoding TNF- α (Rad-hCMV-TNF- α) or FasL (Rad-hCMV-FasL) under the control of the hCMV promoter. Anterior pituitary cells from Wistar female rats were infected with 50 viral particles/cell of Rad-hCMV-TNF- α or Rad-hCMV-FasL for 48 h. The expression of TNF- α (A) and FasL (B) was determined by indirect immunofluorescence (right panels) and nuclei were stained with DAPI (left panels); magnification $\times 400$. (C) A confocal picture shows the expression of FasL in the cytoplasmic membrane of anterior pituitary cells infected with Rad-hCMV-FasL; magnification $\times 600$.

of hypodiploid (Fig. 3A) and TUNEL-positive (Fig. 3B) anterior pituitary cells, whereas the expression of β -Gal did not exert cytotoxic effects (Fig. 3A and B). Figure 3C shows that only anterior pituitary cells successfully infected with RAd-hCMV-TNF- α exhibit apoptotic nuclear morphology.

Proapoptotic effect of TNF- α and FasL transgene expression in murine anterior pituitary cell lines

To determine whether TNF- α and FasL exert an apoptotic effect in tumoral anterior pituitary cells, we infected

the murine anterior pituitary cell lines GH3 and AtT20 with adenoviral vectors encoding these ligands. Since the expression of death receptors has not been previously reported in the somatotrope cell line GH3, we first determined the presence of both TNFR1 and Fas in these cells. We detected strong immunoreactivity for TNFR1 (Fig. 4A, left panel) and Fas (Fig. 4A, right panel) in this cell line. The expression of receptors for TNF- α and FasL in the murine corticotrope cell line AtT20 was previously reported (Kobayashi *et al.* 1997). TNF- α but not FasL immunoreactivity was detected in control cultures of GH3 cells. However, after infection with RAd-hCMV-FasL, these cells showed a strong expression of FasL (Fig. 4B). Although we did not detect TNF- α or FasL immunoreactivity in control AtT20 cells, RAd-hCMV-TNF- α and RAd-hCMV-FasL efficiently transduced these cells (Fig. 4C).

Overexpression of TNF- α and FasL increased the percentage of hypodiploid GH3 cells in a dose-dependent manner, whereas β -Gal expression had no cytotoxic effect even at the highest dose tested (Fig. 5A). Infection of GH3 cells with 50 IU/cell of RAd-hCMV-TNF- α or RAd-hCMV-FasL also increased the percentage of TUNEL-positive cells (Fig. 5A). Overexpression of both vectors in AtT20 cells increased the percentage of hypodiploid cells whereas infection with RAd-hCMV- β -Gal did not induce apoptosis (Fig. 5B). In addition, RAd-hCMV-TNF- α or RAd-hCMV-FasL strongly enhanced the percentage of AtT20 TUNEL-positive cells (Fig. 5B).

Discussion

Previous studies have demonstrated that adenoviral-encoded β -Gal driven under the control of the hCMV promoter can be delivered to anterior pituitary cells in primary culture and into the pituitary cell lines GH3 and AtT20 (Castro *et al.* 1997). Our present results clearly show that adenoviral-encoded TNF- α and FasL induce apoptosis of both normal and tumoral anterior pituitary

Figure 2 Overexpression of TNF- α or FasL induces apoptosis of anterior pituitary cells from Wistar rats. Anterior pituitary cells from Wistar female rats were infected for 48 h with different doses of infectious units/cell (IU/cell) of adenoviral vectors encoding TNF- α , FasL or, as a control, β -Gal, under the control of the hCMV promoter. The percentage of hypodiploid cells was determined by FACS using propidium iodide alone or combined with antibodies against prolactin or growth hormone to detect lactotropes and somatotropes respectively. (A) Each point represents the mean $\pm$ S.E. of the percentage of sub-G1 cells from three wells/group. ** P <0.01 vs mock-infected cells; ANOVA followed by Dunnett's test. (B) Each point represents the mean $\pm$ S.E. of the percentage of sub-G1 lactotropes of three wells/group. ** P <0.01 vs mock-infected cells; ANOVA followed by Dunnett's test. (C) Each point represents the mean $\pm$ S.E. of the percentage of sub-G1 somatotropes of three wells/group. ** P <0.01 vs mock-infected cells; ANOVA followed by Dunnett's test.

Figure 3 Overexpression of TNF- α or FasL induces apoptosis of anterior pituitary cells from Sprague–Dawley rats. Anterior pituitary cells from Sprague–Dawley female rats were infected for 48 h with 50 IU/cell of adenoviral vectors encoding TNF- α , FasL or, as a control, β -Gal, under the control of the hCMV promoter. (A) Nuclei were stained using propidium iodide and the percentage of hypodiploid cells was determined by FACS. Each column represents the mean $\pm$ s.e. of the percentage of sub-G1 cells of three wells/group. ** $P < 0.01$ vs mock-infected cells (Control); ANOVA followed by Dunnett's test. (B) DNA fragmentation was determined by the TUNEL method. Each column represent the percentage $\pm$ confidence limit of TUNEL-positive cells ($n > 1200$ cells/group). ** $P < 0.01$ vs mock-infected cells (Control); χ^2 test. (C) Expression of TNF- α in anterior pituitary cells infected with 50 IU/cell of RAD-hCMV-TNF- α . The expression of TNF- α was determined by immunocytochemistry (left panel) and the nuclei were stained with DAPI (right panel). Photographs show TNF- α immunoreactive cells exhibiting condensed apoptotic nuclei; magnification $\times 1000$.

Figure 4 Expression of TNFR1 and Fas in GH3 cells (A), and of TNF- α and FasL in GH3 (B) and AtT20 (C) cells. (A) The expression of TNFR1 and Fas was determined by immunocytochemistry in control cultures of GH3 cells. (B) GH3 cells were infected with either 50 IU/cell of RAD-hCMV-TNF- α (the expression of TNF- α was determined by immunocytochemistry; upper panels) or 50 IU/cell of RAD-hCMV-FasL (the expression of FasL was determined by indirect immunofluorescence and the nuclei were stained with DAPI; lower panels). (C) AtT20 cells were infected with either 50 IU/cell of RAD-hCMV-TNF- α (the expression of TNF- α was determined by immunocytochemistry; upper panels) or 50 IU/cell of RAD-hCMV-FasL (the expression of FasL was determined by indirect immunofluorescence and the nuclei were stained with DAPI; lower panels). Magnification $\times 200$.

Figure 5 Overexpression of TNF- α and FasL induces apoptosis of somatotrope GH3 cells and corticotrope AtT20 cells. GH3 cells (A) and AtT20 cells (B) were infected for 48 h with different doses of IU/cell or 50 IU/cell of adenoviral vectors encoding TNF- α , FasL or, as a control, β -Gal, under the control of the hCMV promoter. The percentage of hypodiploid cells was determined by FACS (left panels). Each point represents the mean $\pm$ s.e. of the percentage of sub-G1 cells of three wells/group. ** $P < 0.01$ vs mock-infected cells; ANOVA followed by Dunnet's test. DNA fragmentation was determined by the TUNEL method (right panels). Each column represents the percentage $\pm$ confidence limit of TUNEL-positive cells ($n > 1300$ cells/group). ** $P < 0.01$ vs mock-infected cells (Control); χ^2 test.

cells. Considering that the adenoviral vector encoding β -Gal did not exert a cytotoxic effect in any of the cell types studied in our experimental conditions, the present results indicate that the proapoptotic effect of the virus encoding TNF- α or FasL is specifically due to the expression of these proapoptotic factors.

Previous reports indicate that anterior pituitary cells produce TNF- α under basal conditions (Gatti & Bartfai 1993, Goujon *et al.* 1996). Although we found cells expressing TNF- α in control anterior pituitary cultures, hCMV promoter was effective in driving the expression of TNF- α within anterior pituitary cells, increasing TNF- α immunoreactivity. Two different types of receptors for TNF- α have been described: TNFR1 (55 kDa) and TNFR-2 (75 kDa). Although both receptors are coexpressed in the majority of cells, each one can be predominant in specific cell types (Ksontini *et al.* 1998). Only TNFR1 presents an intracellular death domain that triggers an apoptotic cascade (Aggarwal & Natarajan 1996). Both TNFR1 and TNFR-2 are expressed in the mouse anterior pituitary gland (Lewis *et al.* 1991, Wolvers *et al.* 1993) but, in the pituitary of the rat only one binding

site was detected for TNF- α (Watanobe & Yoneda 2003), with an affinity compatible with that of TNFR1 (Lewis *et al.* 1991, Wolvers *et al.* 1993). The cell type/s of the anterior pituitary that expresses this death receptor has not been identified yet. However, our previous results have shown that TNF- α induces apoptosis of lactotropes and somatotropes from female rats (Candolfi *et al.* 2002, 2005) suggesting that at least these cell subpopulations express TNFR1. In fact, our present data show that overexpression of TNF- α in primary anterior pituitary cell cultures induced apoptosis of prolactin- and growth hormone-bearing cells. We previously detected the expression of FasL and Fas in several anterior pituitary cell types from female rats, mainly in lactotropes and somatotropes (Jaita *et al.* 2005b). In addition, we reported that Fas activation induces apoptosis of lactotropes (Jaita *et al.* 2005b) and somatotropes as well (Jaita *et al.* 2005a). Our present results show that viral vectors encoding FasL exert very potent apoptotic effects in lactotropes and somatotropes. Although we cannot rule out that TNF- α or FasL could also induce apoptosis in other anterior pituitary subpopulations, our previous studies showed that TNF- α does not

exert apoptotic effects in gonadotropes and corticotropes (Candolfi *et al.* 2002).

This is the first report demonstrating that the somatotrophic tumor GH3 cell line expresses both TNFR1 and Fas. In the corticotrophic tumor AtT20 cell line, expression of both death receptors has already been reported (Kobayashi *et al.* 1997, Huang *et al.* 2005). In GH3 and AtT20 cells, overexpression of either TNF- α or FasL strongly enhanced cell death by apoptosis, suggesting that death receptor-apoptotic pathways are conserved in both tumoral cell lines. Since GH3 and AtT20 cells express TNFR1 and Fas, TNF- α or FasL gene transfer into transformed pituitary cells may become a feasible therapeutic approach for the treatment of pituitary tumors.

Successful transgene delivery to the anterior pituitary gland has been achieved using first-generation recombinant adenoviral vectors (Castro *et al.* 1997, Windeatt *et al.* 2000, Lee *et al.* 2001a,b, Southgate *et al.* 2001, Williams *et al.* 2001). Although the safety of intrapituitary administration of adenoviral vectors remains controversial (Davis *et al.* 2001, Southgate *et al.* 2001, Carri *et al.* 2005), several transgenes have been shown to be promising gene therapy targets for pituitary adenomas: such as HSV Type 1 thymidine kinase (HSV1-TK) (Lee *et al.* 1999, Windeatt *et al.* 2000), tyrosine hydroxylase (Williams *et al.* 2001) and the diphtheria toxin gene (Lee & Jameson 2002) – all of which have been shown to reduce pituitary growth and hyperprolactinemia in murine pituitary tumor models (Lee & Jameson 2005). Considering that the TNFR1/TNF- α and Fas/FasL systems can act in an auto/paracrine manner, one advantage of TNF- α or FasL gene transfer is that if not all the tumor cells are successfully transduced, the transduced cells, even if few in number, could induce apoptosis in the neighboring cells. Although this could result in toxic effects on normal surrounding tissue, the low diffusibility of replication-defective adenoviral vectors throughout the pituitary gland (Davis *et al.* 2001, Southgate *et al.* 2001, Carri *et al.* 2005) ensures the expression of the transgene only inside the tumor mass. We previously described the low toxicity of RAd-hCMV-FasL in the brain (Ambar *et al.* 1999), which was neither associated with local morphologic changes (such as apoptosis of brain parenchyma cells) nor with systemic alterations; suggesting that the overexpression of death receptor ligands in the pituitary would constitute a safe approach for the treatment of pituitary adenomas. Nevertheless, adenoviral vectors can be engineered to have even lower toxicity. The incorporation of pituitary hormone promoters (Castro *et al.* 1997, Lee *et al.* 2000, 2001b, Davis *et al.* 2001, Southgate *et al.* 2001) into viral vectors has shown to drive transgene expression selectively in specific endocrine pituitary cell populations *in vivo* (Southgate *et al.* 2001, Roche *et al.* 2004). On the other hand, inducible promoter systems driving transgene expression have allowed for tight regulation of transgene

expression in the pituitary gland (Smith-Arica *et al.* 2001, Williams *et al.* 2001).

In conclusion, considering that apoptosis plays a central role in regulation of pituitary tissue homeostasis, the imbalance between cell death and proliferation in favor of cell survival could result in tumor formation. One of the commonly employed strategies in experimental gene therapy for cancer is to target death receptors specifically to trigger apoptosis in tumor cells (Bianco *et al.* 2003, Nakanishi *et al.* 2003, Rubinchik *et al.* 2003, Sanlioglu *et al.* 2004). Our results indicate that forced overexpression of TNF- α or FasL induces apoptosis of normal and tumoral pituitary cells and suggest that TNF- α and FasL gene transfer may be suitable to promote apoptosis of TNFR1 or Fas positive pituitary tumoral cells.

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