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The activity and proliferation of pituitary prolactin-positive cells and pituitary VIP-positive cells are regulated by interleukin 6

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Summary. Interleukins are proteins involved in the immune system and have been related to the endocrine regulation of the hypothalamic-pituitary-adrenal axis as well as to the secretion of ACTH, prolactin (PRL), GH and, possibly, LH. Like interleukin-6 (IL-6), vasoactive intestinal peptide (VIP) is synthesized in the pituitary gland and stimulates prolactin secretion. The aim of the present study was to address whether Interleukin 6 is involved in the regulation of VIP, as well as other factors involved in the regulation of prolactin such as dopamine, TRH and estradiol. Accordingly, we performed an *in vitro* study on monolayer cultures of rat pituitary cells, neutralizing the possible paracrine effect of IL-6 by immunosuppressing the protein by treatment with polyclonal antibody against IL-6 over 1, 3, 6 or 24 hours and then determining the degree of proliferation of VIP cells using double immunocytochemical labelling for VIP or PRL and proliferating cell nuclear antigen (PCNA). As a control, the effects of immunosuppression on the proliferation of PRL-positive cells were analyzed. Immunosuppression of IL-6 induced modifications in the cellular and nuclear size of VIP-positive cells, indicating an inhibitory process. Moreover, immunosuppression induced a significant decrease in the proliferation rate of PRL-positive or VIP-positive cells for all time-points analyzed. Similar effects on the proliferation rate of PRL-positive cells were found. The results of the present study demonstrate that IL-6 is involved in the regulation of the activity and proliferation of pituitary VIP-producing cells and suggest that, without ruling out a direct effect of IL-6 on prolactin cells, IL-6 could regulate prolactin by acting on pituitary VIP.

Key words: VIP-producing cells, Prolactin-producing cells, Pituitary gland, Interleukin-6, Immunosuppression, Cellular proliferation, Cell cultures

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Introduction

The pituitary gland displays an auto-paracrine regulation that is not fully understood and that involves peptides produced and released by the gland itself (Li et al., 1984; May et al., 1987; Joubert et al., 1989; Hsu et al., 1989; Pagesy et al., 1989; Segerson et al., 1989; Peillon et al., 1990; Lam, 1991; Carretero et al., 1991).

Although its functions remain to be fully elucidated it is known that these peptides mediate the regulation of the pituitary hormone secretion induced by hypothalamic agents and peripheral hormones, perhaps participating in cell proliferation processes, and their expression can be modulated by hypothalamic factors and peripheral hormones.

The pituitary gland also produces growth factors and cytokines that are involved in the regulation of hormone secretion and pituitary cell proliferation and, additionally, they modulate or induce phenotypic transformations in certain cell populations (Binnerts et al., 1990; Akutsu et al., 1991; Driman et al., 1992;

Sarkar et al., 1992; Artz et al., 1993; Atkin et al., 1993; Missale et al., 1993; Carretero et al., 1999).

Although some interleukins have been implicated in the regulation of cell proliferation, as may be seen in the studies of Artz et al. (1993, 1995), the true physiological meaning of this regulation is unknown and, likewise, it is not known how they affect VIP-producing pituitary cells, which regulate the functionality of the gland in an auto-paracrine fashion.

In previous studies, we observed that interleukin-6 is expressed in the pituitary gland (Moro et al., 2008) and it modulates the *in vitro* apoptosis and cellular proliferation of pituitary cells (Carretero et al., 1999).

Since VIP is an auto-paracrine regulator of pituitary prolactin, and since it is known that VIPomas may occur inside prolactinomas, that VIPergic cells respond to factors that typically regulate prolactin, such as estradiol, dopamine and testosterone, and that IL-6 is involved in the regulation of this hormone, in light of the data reported above we were prompted to assess whether IL-6 modulates the pituitary VIPergic population or not.

To address this issue, we carried out *in vitro* IL-6 immunosuppression studies and explored its effects on the morphology and proliferation, determined by means of the intranuclear expression of PCNA, of VIPergic cells and lactotroph cells identified by immunocytochemical expression of the peptide or the hormone present in monolayer cultures of pituitary cells. We also performed a morphometric evaluation of VIPergic cells in the cultures.

Materials and methods

Pituitary cultures

Following anaesthesia with Forene®, male Wistar rats (175-200 g, 8-10 moths old) were killed by decapitation (the animals were handled according to the guidelines of the European Communities Council Directive: 86/609/EEC and current Spanish legislation for the use and care of laboratory animals: BOE 67/8509-12, 1998), the primary pituitary cultures were carried out according to the protocol previously described by our laboratory (Carretero et al., 1997): 1. The anterior pituitary glands were removed and washed in Earle's balanced salt solution; 2. Enzymatic dispersion was accomplished by incubation for 15 minutes at 37°C in Hank's solution to which 0.15% $MgCl_2$, 0.1% papain, 0.01% DNase and 0.1% neutral protease had been added; 3. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20 to 22 gauge needles; 4. After centrifugation, the supernatant was removed and the cells were resuspended in an appropriate volume of Dulbecco's modified Eagle's medium, supplemented with 10% calf serum, 2.5% foetal calf serum, 2% L-glutamine, 1000 IU/ml of penicillin and 1000 IU/ml of streptomycin; 5. The cells were seeded on culture chamber slides at a final concentration of $2x10^5$ cells/dish and incubated at 37°C in a 5% CO_2/95% air atmosphere for 7 days, and 6. On the 4th day of incubation the medium was replaced by fresh medium.

Immunosuppression of IL-6

On the 7th day of incubation, the medium was replaced by fresh medium with non-specific rabbit serum (Dako® 1:100) in control dishes or fresh medium with polyclonal rabbit anti-rat IL-6 serum (Endogen®, 10 μg/ml) in treated dishes and incubated for 1, 3, 6, or 24 hours. Five dishes per treatment, PRL- or VIP-labelling, and time-point assayed were employed. At the end of each experiment, the dishes were carefully washed with Dulbecco's sterile PBS and the cells were fixed in 15% picric acid in 4% paraformaldehyde in phosphate buffer (0.01M, pH7.4) for 30 min, followed by careful rinsing in PBS (the concentration of IL-6 Ab for immunosuppression was indicated by the manufacturer).

Immunocytochemistry

To study PCNA- and VIP- or PRL-positive cells, a double-labeling immunocytochemical method for PCNA and VIP or for PCNA and PRL was developed. The first labeling applied was for PCNA and then for VIP or for PRL.

In both cases, PRL or VIP, after blocking endogenous peroxidase with methanol and H_2O_2 and non specific reactions of the secondary antibody by incubation in normal goat serum (1:30) in TBS (Tris-saline buffer 0.05M, pH 7.6 in 0.8% of NaCl and 0.1% of Triton X-100) the cells were incubated overnight at 4°C with the mouse PC10 mAb (Dako, lot. 21, diluted 1:3000 in TBS). Biotinylated goat anti-mouse IgG (Dako, lot.061, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit, Dako, lot. 081, diluted 1:100) were successively applied at room temperature for 40 minutes and 30 minutes, respectively. The reaction was developed in freshly prepared 3-3'DAB (0.25% in TRIS buffer containing 0.03% of H_2O_2).

Following the PCNA immunolabeling, the peroxidase-antiperoxidase (PAP) reaction was performed for the detection of PRL or VIP by overnight incubation with rabbit antiserum against PRL (Dako lot. 042a, diluted 1:1000) or rabbit antiserum against VIP (kindly supplied by Dr. Sánchez-Franco, ISCIII, Madrid, Spain; diluted 1:1000) at 4°C. Swine anti-rabbit IgG (Dako lot. 022, diluted 1:200) and rabbit PAP soluble complex (Dako lot. 021, diluted 1:200) were successively applied at room temperature for 30 minutes. The reaction was developed using 4-chloro-1-naphthol ($1.7x10^{-3}$ M in 3% absolute ethanol and TRIS-buffer containing 0.3% H_2O_2).

Method controls included substitution of the primary antibodies with normal, mouse or rabbit, serum or TBS; preabsorption tests with PRL or VIP, as well as omission of the secondary antibody or ABC and PAP complex. The specificity of swine anti-rabbit serum was tested by

substitution and preabsorption tests with mouse IgG. After these tests, no immunoreactivity was detected. The cross-reaction with mouse or rat immunoglobulins was determined by ELISA and was also very low, less than 1%. The anti-VIP serum has been tested previously for radioimmunoassay, immunocytochemistry or immunoblockade at our laboratory (Carretero et al., 1994, 1995a, 1996, 1997, 1998, 2003, 2006).

Quantification of VIP- and PCNA-VIP-positive cells, or PRL- and PCNA-PRL-positive cells

Four thousand cells per dish and study were evaluated using an Axioplan Zeiss® microscope equipped with an ocular grid at a final magnification of x 400. The cells were randomly selected from different areas of the dishes. Dishes with a high degree of agglomeration of overlapping cells were rejected and only non-overlapping cells were considered. Because the PCNA protein is known to have a 20-hour half-life in *in vitro* systems (Bravo et al., 1987) and is detected in cells that have recently completed mitosis (Hall et al., 1990; Coltrera and Gown, 1991), only nuclear PCNA expression was considered, except in metaphasic-mitotic cells. The following parameters were determined: number of VIP-positive cells, number of PCNA-VIP-positive cells, number of PRL-positive cells, number of PCNA-PRL-positive cells; these parameters were calculated as percentages of the total number of cells analyzed. The percentages of PCNA-VIP-positive cells from the VIP-positive cells, and the percentages of PCNA-PRL-positive cells from the PRL-positive cells were also calculated.

Morphoplanimetry

Using an Apple digital planimeter connected via an RCA video system to a Leitz Dialux EB-20 microscope and the Image J (NIH application), a total of 1000 VIP-positive cells per group of treatment was analyzed, calculating in each dish the cellular area of 200 VIP-reactive cells chosen at random from the surface of the dishes.

Statistical analyses

Two pituitary cultures under similar experimental conditions were performed in order to verify the reproducibility of the results (the within-assay error was less than 1.96%). For each parameter evaluated (n=20), the values obtained were subjected to ANOVA followed by the Scheffé F test for multiple comparisons, accepting $p<0.05$ as significant. The results are expressed as arithmetic means ± SEM.

Results

Proliferative ratio of PRL-immunopositive cells

Figs. 1a-c show the pattern of reaction of PCNA- and PRL-positive cells, brown nuclear staining for PCNA, and dark blue cytoplasmic staining for PRL.

The percentages of PRL-positive cells are summarized in the Fig. 2a. To start the experiment (0 hours) it was 31.81±0.63%. From 1 to 24 hours in culture, the percentages of PRL-positive cells in the control dishes were very similar and no significant differences were found. After immunosuppression with Ab-IL6, on comparing the results with the same control time-points, significant decreases in the percentages of PRL-positive cells, $p<0.01$, were found, except for 1 hour of treatment (1 hour: 28.93±0.15 vs. 31.01±0.21; 3 hours: 21.14±0.33 vs. 32.24±0.21; 6 hours: 21.51±0.35 vs. 31.27±0.20; 24 hours: 25.70±0.17 vs. 33.31±0.31).

When the percentages of PCNA- and PRL-positive cells of the total cells present in the cultures were analyzed (Fig. 2b), no significant differences in the control time-points were found. However, after immunosuppression with Ab-IL6 significant differences ($p<0.01$) were found for all time-points assayed (1 hour: 11.26±0.15 vs. 17.45±0.33; 3 hours: 10.17±0.45 vs. 18.90±0.78; 6 hours: 10.01±0.39 vs. 18.87±0.66; 24 hours: 12.69±0.81 vs. 19.60±0.57).

Similar findings were found when only PRL-positive cells were considered (Fig. 2c). The percentages of PCNA- and PRL-positive cells of the total PRL-positive cells were very similar in all control time-points assayed, but significant differences ($p<0.05$) were found following immunosuppression with Ab-IL6 (1 hour: 49.11±1.35 vs. 58.15±1.45; 3 hours: 48.11±1.22 vs. 58.62±1.55; 6 hours: 46.54±0.91 vs. 60.35±1.56; 24 hours: 45.49±1.33 vs. 58.84±1.48).

Proliferative ratio of VIP-immunopositive cells

Figures 1d, e and f show the reaction pattern of PCNA- and VIP-positive cells: brown nuclear staining for PCNA, and dark blue cytoplasmic staining for VIP.

The percentages of VIP-positive cells are summarized in Fig. 2d. To start the experiment (0 hours) it was 29.22±0.48%. From 1 to 24 hours in culture, the percentages of VIP-positive cells in the control dishes were very similar and no significant differences were found. After immunosuppression with Ab-IL6, On comparing the results with o the same control time-points, significant decreases in the percentages of VIP-positive cells, $p<0.05$, were found, except for 1 and 3 hours of treatment. These decreases were more evident at the highest time-points of immunosuppression (1 hour: 28.72±0.17 vs. 28.74±0.20; 3 hours: 25.77±0.19 vs. 27.84±0.14; 6 hours: 24.13±0.16 vs. 28.10±0.14; 24 hours: 24.96±0.17 vs. 31.98±0.21).

After double immunocytochemical labelling for PCNA and VIP, around 8% of the pituitary cultured cells at the control time-points were PCNA- and VIP-positive cells (Fig. 2e). These did not display significant modifications at any of the four times studied. Following immunosuppression with Ab-IL6 significant decreases in this percentage ($p< 0.01$) were observed at 3, 6 and 24 hours (1hour: 7.17±0.47 vs. 8.15±0.76; 3 hours:

4.78±0.27 vs. 8.29±0.68; 6 hours: 3.03±0.39 vs. 8.78±0.83; 24 hours: 3.71±0.41 vs. 9.99±0.54).

When only VIP-positive cells were considered, in control dishes no significant differences among the time-points assayed were found (Fig. 2f): 1 hour: 28.36±0.99, 3 hours: 29.78±1.04, 6 hours: 31.23±1.09, 24 hours: 31.25±1.07. IL-6 immunosuppression altered the percentage of VIP-positive cells undergoing proliferation to a significant extent at all times studied (Fig. 2f). The decrease became more intense when the time of immunosuppression was prolonged: 1 hour: 23.43±0.82, 3 hours: 18.56±0.65 ($p<0.01$ with respect to 1 hour) and

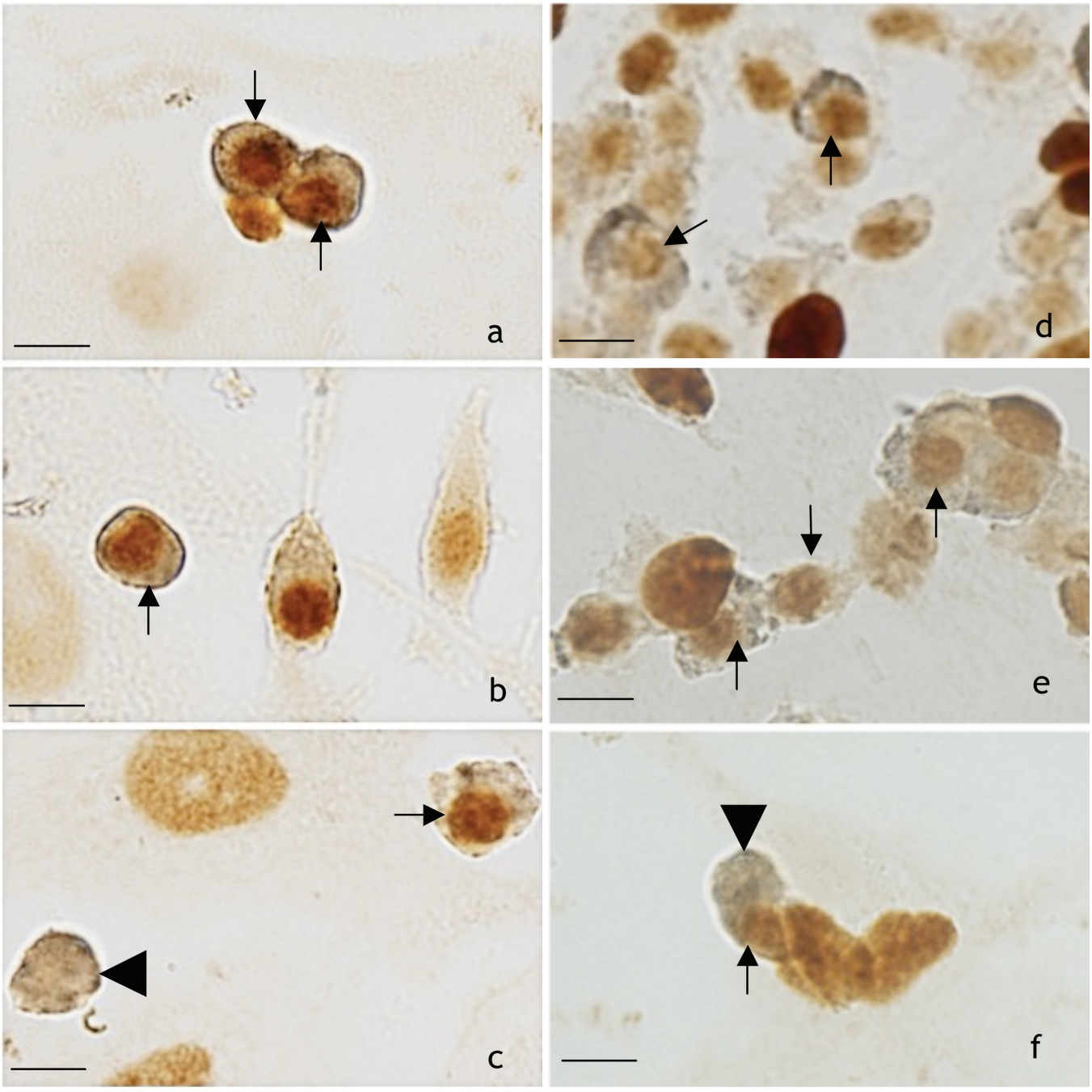

Fig. 1. Micrographs obtained from pituitary cultures. **a.** PCNA- and PRL-positive cells (arrows) from a control culture after 3 hours of incubation. **b.** PCNA- and PRL-positive cell (arrow) from a culture after 3 hours of immunosuppression of IL6. **c.** PCNA- and PRL-positive cell (arrow) and PRL-positive cell (arrow head) observed in a culture after 24 hours of immunosuppression of IL6. **d.** PCNA- and VIP-positive cell (arrow) from a control culture after 3 hours of incubation. **e.** PCNA- and VIP-positive cell (arrow) from a culture after 3 hours of immunosuppression of IL6. **f.** PCNA- and VIP-positive cell (arrow) and VIP-positive cell (arrow head) from a culture after 6 hours of immunosuppression of IL6. Scale bars: a-c, e, f, 20 μm, d, 30 μm.

6 hours: 12.54±0.48 ($p<0.01$ with respect to 1 and 3 hours), thereafter recovering slightly at 24 hours 14.87±0.52 ($p<0.05$ with respect to 3 and 6 hours, and $p<0.01$ with respect to 1 hour).

The effect of IL-6 immunosuppression was very pronounced upon comparing each immunosuppression time with each of its control wells. After 1 hour of IL-6 immunosuppression, the percentage of VIP-positive cells undergoing proliferation decreased significantly, by 17% (23.43±0.82 vs. 28.36±0.99, $p<0.05$). This effect

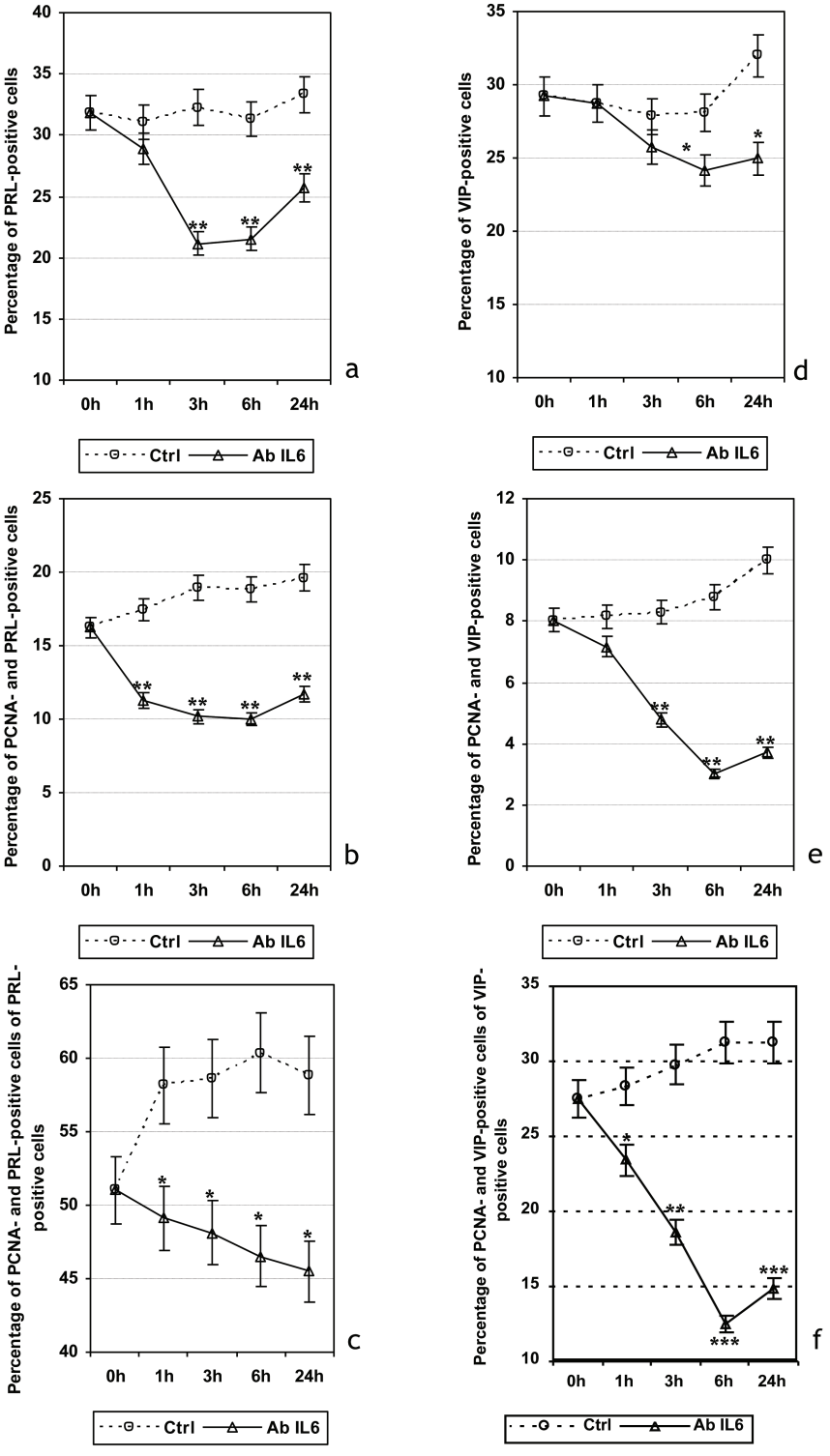

Fig. 2. Plots showing the effects of the different time-points of immunosuppression on the percentages of PRL- or VIP-producing cells. **a.** Percentages of PRL-positive cells. *: $p<0.01$ with respect to their controls. **b.** Percentages of PCNA- and PRL-positive cells. *: $p<0.01$ with respect to their controls. **c.** Percentages of PCNA- and PRL-positive cells of total PRL-positive cells. *: $p<0.05$ with respect to their controls. **d.** Percentages of VIP-positive cells. *: $p<0.05$ with respect to their controls. **e.** Percentages of PCNA- and VIP-positive cells. *: $p<0.01$ with respect to their controls. **f.** Percentages of PCNA- and VIP-positive cells of total VIP-positive cells. *: $p<0.01$ with respect to 1 hour of immunosuppression and to their control. **: $p<0.05$ with respect to their control. #: $p<0.01$ with respect to 1 and 3 hours of immunosuppression and $p<0.005$ in relation to their control. ##: $p<0.005$ with respect to their control, $p<0.05$ with respect to 3 and 6 hours and $p<0.01$ with respect to 1 hour of immunosuppression.

intensified at 3 hours (18.56±0.65 vs. 29.78±1.04, p<0.01) and even more so at 6 hours (12.54±0.48 vs. 31.23±1.09, p<0.005), remaining steady at 24 hours (14.87±0.52 vs. 31.25±1.07, p<0.005).

Morphological changes after immunosuppression

VIP-positive cells appeared in the dishes isolated or grouped in small islets. They were polygonal or oval cells. In control dishes, VIP-positive cells usually showed a compact and peripheral dark blue or grey cytoplasmic staining, with a perinuclear negative space (Fig. 1d). After immunosuppression, the intensity of the cytoplasmic stain decreased with respect to the control dishes; the immunocytochemical reaction was granular and was distributed across the whole surface of the cytoplasm. The negative perinuclear space was not present after immunosuppression (Fig. 1e). In the case of prolonged immunosuppression (Fig. 1f) small adjacent cells were frequently found as proliferative cells (arrow in Fig. 1f) or non-proliferating cells (arrowhead in Fig. 1f). The immunocytochemical stain was distributed throughout the cytoplasm but it was not a granular reaction.

Morphometric changes

Along the times studied, the cellular and nuclear areas of the VIP-positive cells in the control cultures increased discretely but significantly.

As can be seen in Fig. 3a, in the control cultures, cell size (cellular area) increased significantly (p<0.01) from hour 1 (105.12±3.68 μm^2) to 24 hours (116.88±4.09 μm^2), with no significant differences between hour 1 and hour 3 (108.18±3.79 μm^2) and hour 6 (108.02±3.78 μm^2), although they were seen from 6 to 24 hours (p<0.05).

In these cultures, the nuclear areas (Fig. 3b) also increased significantly (p<0.01) from hour 1 (28.53±1.00 μm^2) to hour 24 (35.50±1.24 μm^2). In this case, the increase was progressive from hour 1 to hour 3 (30.54±1.07 μm^2), from hour 3 to 6 (32.02±1.12 μm^2), and from hour 6 to 24 hours, although the differences were not statistically significant.

Immunosuppression of IL-6 present in the cultures elicited important changes in both parameters (Fig. 3a, 3b), which depended on the duration of the immunosuppression.

These changes were significantly different upon comparing the times of immunosuppression, as occurred upon comparing each of the times with the respective control wells.

Cell size showed similar values after 1 hour (115.52±4.04 μm^2) and 3 hours (116.61±4.08 μm^2) of immunosuppression. At 6 hours after beginning treatment, the size (95.37±3.34 μm^2) of the VIP-positive cells decreased significantly (p<0.01). This decrease in size began to recover significantly (p<0.05, with respect to all the immunosuppression times studied) at 24 hours after starting the treatment (107.49±3.76 μm^2).

Unlike what occurred with the whole set of cells, the nuclei of the VIP-positive cells increased in size progressively (1 hour: 23.50±0.83 μm^2, 3 hours: 26.02±0.91 μm^2, 6 hours: 27.51±096 μm^2, 24 hours: 34.32±1.20 μm^2) and significantly (p<0.001) from the start to the end of the treatment, with no significant differences between 3 and 6 hours, although the intermediate differences were significant (1 vs. 3 hours: p<0.05; 6 vs. 24 hours: p<0.01).

On comparing the effects of immunosuppression with the control wells, we observed that cell size was substantially altered and inverse, depending on the time of immunosuppression, with two points of inflection between 3 and 6 hours and 6 and 24 hours.

After 1 and 3 hours of immunosuppression a significant increase (p<0.01) in cell size occurred (1 hour: 115.52±4.04 μm^2 vs. 105.12±3.68 μm^2; 3 hours: 116.61±4.08 μm^2 vs. 108.18±3.79 μm^2).

By contrast, after 6 hours the cellular area was significantly smaller (p<0.01) than that observed in the

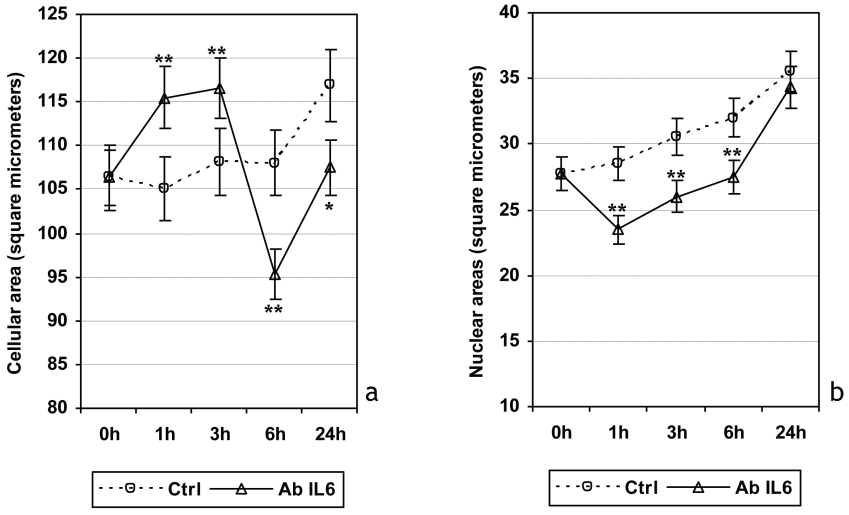

Fig. 3. Morphometric variations according to the different time-points of immunosuppression of IL6. **a.** Cellular areas of VIP-producing cells in square micrometers. *: p<0.01 with respect to 1 and 3 hours of immunosuppression and p<0.01 with respect to their control. **: p<0.05 with respect to 1 and 3 hours of immunosuppression, p<0.05 with respect to its control and p<0.01 in relation to 6 hours of immunosuppression. #: p<0.01 with respect to their control. **b.** Nuclear areas of VIP-producing cells in square micrometers. *: p<0.01 with respect to their controls.

control wells (95.37±3.34 μm^2 vs. 108.02±3.78 μm^2).

At 24 hours of immunosuppression, although the values were significantly lower ($p<0.05$) than in the control wells (107.49±3.76 μm^2 vs. 116.88±4.09 μm^2), the cellular area was significantly larger than at 6 hours of immunosuppression ($p<0.01$).

Unlike the cellular areas, the nuclear areas were always smaller after the immunosuppression of IL-6 than in the respective control wells, similar significant differences being observed after 1 hour (23.50±0.83 μm^2 vs. 28.53±1.00 μm^2, $p<0.01$; 26.02±0.91 μm^2 vs. 30.54±1.07 μm^2, $p<0.01$; 27,51±0.96 μm^2 vs. 32.02±1.12 μm^2, $p<0.01$). At 24 hours, although the values found were lower than in the control wells the differences were not significant (34.32±1.20 μm^2 vs. 35.50±1.24 μm^2).

Discussion

VIP, synthesized (Arnaout et al., 1986; Segerson et al., 1989; Lam et al., 1990; Lam, 1991), released (Sánchez et al., 1991) and located (Besson et al., 1979; Samson et al., 1979; Rosztejn et al., 1980; Morel et al., 1982; Köves et al., 1990; Carretero et al., 1994, 1995b, 1997, 1998; Carrillo y Phelps, 1992) in the pituitary gland, stimulates prolactin secretion (Köves et al., 1990; Chiodera et al., 1996), partly through cAMP-dependent protein kinases (Kansaku et al., 1998).

Inhibitors of pituitary VIP secretion, such as testosterone or dopamine, decrease the size and proliferation of VIP-positive cells. The retention of the peptide inside cells that accompanies the decrease in the release of VIP elicits an initial increase in the intensity of the cytoplasmic reaction (Carretero et al., 1997, 1998), the opposite effects being observed after stimulation with estradiol (Carretero et al., 1995b, 1998).

In the present study we report a similar *in vitro* effect for PRL and VIP, although the effects of immunosuppression of IL6 on cellular proliferation were more evident in VIP than in PRL. Because VIP regulates the proliferation of PRL pituitary cells, the effects of IL6 on PRL could be mediated by its effects of VIP.

Our study shows that IL-6 immunosuppression causes significant changes in cell size, affecting the nuclear and cytoplasmic areas. With the exception of differences in the reaction intensity and cell size, no other morphological differences were observed in the VIP-positive cells. Initially, following immunosuppression an increase occurred in the cell area at the expense of an increase in the cytoplasmic area, whereas the nuclear area decreased. After 6 hours of immunosuppression, the inhibitory effects became more evident, since together with a greater decrease in the nuclear area we observed a decrease in the cellular area. With the exception of the cells undergoing proliferation, at 24 hours of treatment all the parameters analyzed were close to those found in the control cultures.

Detailed analyses addressing the percentage of pituitary cells labelled for VIP are scarce and have mainly been reported for *in vitro* studies. The percentages obtained in the present work are consistent with the results of earlier *in vitro* studies carried out on VIP-positive cells (Carretero et al., 1995b, 1996, 1997, 1998).

The percentages of each type of pituitary cell are different in the adult rat (Dada et al., 1983, 1984), but an important aspect is that the percentages in this species vary, depending on the general endocrine status, and each cell type proliferates in response to hormones and hypothalamic factors (Jacobi et al., 1977; Romano et al., 1984; Sakuma et al., 1984; Inoue et al., 1985, 1986; Perez et al., 1986; McNicol et al., 1990; Zhou-Li et al., 1992; Arola et al., 1993; Oishi et al., 1993). In general, the proliferating cell fraction in the anterior pituitary gland of adult animals is low (Pomerat, 1941; Hunt, 1943; Städtler et al., 1970; Stepién et al., 1978), whereas it is high during development (Shirasawa and Yashimura, 1982; Takahashi et al., 1984; Carbajo et al., 1989; Carbajo-Pérez and Watanabe, 1990) and in *in vitro* studies (Carretero et al., 1995a). These percentages vary in response to three main cellular phenomena: differentiation, proliferation and apoptosis.

The three processes are complex, and in them hormonal stimulation and inhibition play an important regulatory function (Asa, 1991). As well as peptides and hormones, the pituitary gland synthesizes growth factors and cytokines that could be involved in the auto-paracrine regulation of hormone secretion and in pituitary cell proliferation through the modulation or induction of phenotypic transformations of different cell populations (Binnerts et al., 1990; Sarkar et al., 1992; Driman et al., 1992; Missale et al., 1993; Artz et al., 1993; Renner et al., 1997).

IL-6 is produced in tumoral and non-tumoral hypophyses (Vankelecom et al., 1989; Jones et al., 1991, 1993, 1994; Velkeniers et al., 1994; Green et al., 1996; Allaerts et al., 1997; Borg et al., 2003; Moro et al., 2008) and is involved in the regulation of pituitary hormone secretion (Spangelo et al., 1989); moreover, all classical endocrine pituitary cells have receptors for several interleukins (Artz et al., 1995).

In a previous study, we showed that the *in vitro* immunosuppression of IL-6 induces a decrease in the proliferation and an increase in the apoptosis of pituitary cells, suggesting that IL-6 could act as an auto-paracrine regulator of both processes (Carretero et al., 1999).

Here we observed that IL-6 immunosuppression led to a significant decrease in the percentages of cultured pituitary cells that expressed intranuclear PCNA and modified the proliferation of PRL-positive and VIP-positive cells at all times studied to a substantial extent, although more strikingly so at 6 hours after the start of immunosuppression.

Since VIP can be synthesized in more than one type of pituitary cells, it is debatable which of these types of cells will be affected by the immunosuppression of IL-6. VIP-positive cells can be found in the pituitary gland coexisting with different hormone-producing cells, depending on the endocrine physiological, experimental

or pathological status. VIP-immunoreaction has been described in lactotroph cells (Morel et al., 1982) and their mRNA (Chew et al., 1996). These cells may be VIP-positive under basal conditions (Noguchi et al., 1988) or after stimulation with estradiol (Köves et al., 1990). However, in the literature coexistence of VIP in other pituitary cells different from lactotrophs has been described (Carrillo and Phelps, 1992). Moreover, the coexistence for VIP and FSH in gonadotroph pituitary cells from peritumoral tissues in human pituitary adenomas associated with Cushing's disease has been reported (Reyes et al., 2007).

The morphology and percentages of PRL-positive and VIP-positive cells described in this study are different, suggesting that without excluding the possibility that some VIP-positive cells could be also PRL-positive cells other pituitary cells able to exert paracrine regulation on lactotroph cells, such as gonadotroph cells (Denef, 2008), could be affected by the immunosuppression of IL-6.

Nevertheless, because the autocrine or paracrine role of VIP in the pituitary gland mainly involves the regulation of prolactin cells, and pituitary VIP-producing cells respond in a similar way to prolactin to physiological endocrine regulators (Carretero et al., 1996, 1997, 1998, 2003), it is more important to determine whether VIP might be one of the pathways involved in the effects of IL-6 on prolactin, as shown in this study, in a similar way what has been demonstrated for estradiol (Prysor-Jones et al., 1988; Carretero et al., 1995b), than to determine whether the effects of the immunosuppression of IL-6 occur in hormonal or non-hormonal pituitary VIP-producing cells.

Interleukin-6, whose regulating effects on pituitary VIP is demonstrated here, participates in the endocrine regulation of the pituitary gland (Spangelo et al., 1989; Harbuz et al., 1992) and acts on the proliferation of lactotroph cells (Artz et al., 1993). This issue is of huge importance since pituitary VIP has as its main function the stimulation of prolactin synthesis and release (Kato et al., 1978; Rotsztejn et al., 1980; Lam and Reichlin, 1989), and it can play a role as a growth factor for lactotroph cells in pituitary tumours (Fazekas et al., 2000) and in the non-tumoral pituitary gland (Carretero et al., 2006).

Because IL-6 is able to increase the proliferation of lactotroph cells (Artz et al., 1993) and since in the present study we demonstrate that *in vitro* immunosuppression of IL-6 decreases the proliferation of this type of pituitary cells, in a similar way, the decreases in the percentages of cells reactive to VIP and PCNA observed after IL-6 immunosuppression suggest the existence of a mitogenic role, *in vivo*, of interleukin in cells immunoreactive to the peptide.

Together, these results suggest that IL-6 and VIP could be two elements of the same regulatory pathway in the maintenance of the pituitary lactotroph population and it may well be that part of the action of IL-6 on prolactin-producing cells could be carried out through activation and proliferation of VIP-positive cells.

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