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Effects of in vitro immunosuppression of Interleukin-6 on the proliferation of rat hypophyseal cells

J. Carretero[1,2], A. Martín-Clavijo[2], G. Vázquez[1,2], J. Somalo[2], M. Rubio[1,2], F. Sánchez[2], E. Hernández[1], M.C. Montero[1], J.L. Torres[1] and R. Vázquez[1,2]

1.- Laboratory of Neuroendocrinology, Institute of Neurosciences of Castilla y León. Faculty of Medicine, University of Salamanca. Spain

2.- Department of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca. Spain

Summary

Interleukins –ILs– are proteins involved in the immune system and have been related to the endocrine regulation of the hypothalamic-hypophyseal-adrenal axis as well as to the secretion of ACTH, prolactin, GH and, possibly, LH. IL synthesis has been reported in the hypophysis and the action of these compounds is therefore believed to occur through paracrine mechanisms. IL-6 has been implicated as a regulatory factor involved in pituitary cellular proliferation. However it is not determined whether the IL-6 proliferative effects are paracrine effects. The aim of the present work was to address these questions. Accordingly, we performed an in vitro study on rat hypophyseal cells, neutralising the possible paracrine effect of IL-6 by immunosuppressing the protein by treatment with diluted (1:10) polyclonal antibody against IL-6 -Ab-IL-6- over 1, 3, 6 12 or 24 hours and later determining the degree of proliferation, determining the PCNA. In the control dishes, the percentages of PCNA-immunoreactive cells ranged from 43% to 50%, with no significant differences in the different time periods assayed. Immunosuppression of IL-6 induced significant decreases in the percentages of PCNA-immunoreactive cells ($p<0.01$ after 3, 6 and 12 hours). The results of the present study suggest a dual role for IL-6 in the modulation of hypophyseal cells; i.e., stimulating proliferation and inhibiting cellular apoptosis in the rat hypophysis.

Introduction

Hypophyseal cellular differentiation and proliferation are complex processes in which hormonal stimulation and inhibition play an important role (Asa, 1991). Not all hyper- or hypofunctional hypothalamic states or hyper- and hyposecretory states of the peripheral glands necessarily lead to the formation of hypophyseal adenomas, although they do generally induce glandular hyperplasias that affect some of the different cellular populations of the gland. Accordingly, it can be surmised that there must be mechanisms associated with hormonal regulation that are involved in adenomatose dedifferentiation in the hypophysis and that are probably related to transduction signals and the activation of proto-oncogenes (Lloyd et al., 1991).

The hypophysis is subject to auto-paracrine regulation involving peptides produced and released by the gland itself (Li et al., 1984; May et al., 1987; Joubert et al., 1989; Segerson et al., 1989; Peillón et al., 1990; Carretero et al., 1991). The function of these peptides remains obscure, although they are known to participate in the regulation of hypophyseal hormone secretion induced by hypothalamic agents and peripheral hormones and also that they participate in the regulation of cellular proliferation. Additionally, as happens during development, they are involved in the development and maintenance of cellular differentiation.

The hypophysis also produces growth factors and cytokines that could be involved in the auto-paracrine regulation of hormone secretion and of hypophyseal cellular proliferation by modulating or inducing phenotypic transformations in different cellular populations (Binnerts et al.,

Correspondence to:
Prof. Dr. J. Carretero. Laboratorio de Neuroendocrinología, Instituto de Neurociencias de Castilla y León. Facultad de Medicina
Avda. Alfonso X "El Sabio" s/n. 37007 Salamanca, Spain
Fax: +34 23 294559
E-mail: jcar@gugu.usal.es

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1990; Sarkar et al., 1992; Driman et al., 1992; Missale et al., 1993; Artz et al., 1993; Renner et al., 1997; Quan et al., 1998).

In the present study we focused our attention on interleukin-6 (IL-6). The hypophyseal synthesis and release of IL-6 has been well documented (Vankelecom et al., 1989; Jones et al., 1991; 1994; Velkeniers et al., 1994; Green et al., 1996; Spangelo and Jarvis, 1996; Allaerts et al., 1997; Martin-Clavijo et al., 1997; Renner et al., 1997). Additionally, IL-6 has been implicated in hypophyseal endocrine regulation (Spangelo et al., 1989; Harbuz et al., 1992) and reports have also been made of the stimulation of the growth of lactotroph cells and of the inhibition of growth of the rest of the adenohypophyseal cellular population following the action of IL-6 (Artz et al., 1993) as well as the stimulation of the cellular proliferation of rat pituitary clonal cell lines in vitro (Sawada et al., 1995) and folliculostellate-like tumoral cells (Renner et al., 1997).

Although apoptosis has been implicated, together with cellular proliferation, in the modulation of pituitary gland cytology (Drewett et al., 1993; Yin et al., 1993, 1994; Aoki et al., 1998), no studies analysing possible auto-paracrine roles for interleukin-6 in apoptosis in on pituitary gland have been reported.

The aim of the present study was to analyse the paracrine effects of IL-6 on the proliferation and apoptosis of hypophyseal cells by means of an in vitro study on monolayer hypophyseal cell cultures subjected to the immunosuppression of IL-6. Cellular apoptosis in the cultures was determined by means of BrdU hybridization to the 3' ends of the fragmented DNA of apoptotic cells and cellular proliferation was estimated by means of immunocytochemical PCNA expression.

Materials and Methods

Pituitary cultures. Following anaesthesia with Forene®, male Wistar rats (175-200g) were killed by decapitation (animals were handled according to 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) and the anterior pituitary glands were removed and washed in Earle's balanced salt solution. 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. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20 to 22 gauge needles. 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. 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. 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, 12 or 24 hours. Five dishes per treatment, study (proliferation or apoptosis), 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, pH 7.4) for 30 min, followed by careful rinsing in PBS (the concentration of Ab-IL-6 for immunosuppression was kindly indicated by the manufacturer).

Immunocytochemistry for PCNA. To determine PCNA-positive cells, the biotinylated-avidin-peroxidase immunohistochemical method was implemented in a similar fashion to what has been reported previously (Carretero et al., 1995a, 1995b, 1997). Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody were blocked by incubation in normal goat serum (Dako®, diluted 1:30). Cultures were incubated overnight at 4°C with the mouse PC10 (PCNA) mAb (Dako®, diluted 1:3000 in TBS). Biotinylated goat anti-mouse IgG (Dako®, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. Reactions were developed in freshly prepared 3-3'DAB (0.025% in TRIS buffer containing 0.03% of H_2O_2).

BrdU in-situ tailing of fragmented DNA. Apoptotic cells were labelled by in situ DNA end-labelling bromodeoxyuridine (BrdU) method (ISEL), adapted to in vitro studies from the in vivo method of Aschoff et al. (1996). After careful rinsing in 66 mM PBS, cultures were incubated with TdT-reaction mixture (Boehringer, Mannheim®) consisting of 8 µl (200 units) of TdT (Terminal d-Transferase), 8 µl of TdT-reaction buffer, 3 µl of $CoCl_2$ stock solution (25 mM), and 1 µl of BrdU (2.5 mg/ml) in 400µl of 66 mM TBS (8.8 g NaCl, 6.06 g Tris/1000 ml). Cultures were incubated with the reaction mixture at 37°C for 1 hour. Incorporated BrdU was visualised by

immunocytochemistry. After in situ hybridization of BrdU, to study the percentage of BrdU-positive cells the Biot-Stav-Pox immunohistochemical method was implemented. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody by incubation in normal goat serum (Dako®, diluted 1:30). Cultures were incubated overnight at 4°C with the mouse anti-BrdU monoclonal antibody (Dako®, diluted 1:250 in TBS). Biotinylated goat anti-mouse IgG (Dako®, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared 3-3'DAB (0.025% in TRIS buffer containing 0.03% of H_2O_2). Controls included substitution of the primary antibody by normal mouse serum or TBS, as well as omission of the secondary antibody or ABC and PAP complex; after both tests no immunoreactivity was detected. The cross-reaction of swine anti-rabbit antiserum IgG with mouse or rat immunoglobulins was determined by ELISA and was also very low (less than 1%).

Quantification of PCNA- or BrdU-immunoreactive 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 400x. 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. PCNA- or BrdU-immunoreactive cells were determined and the percentages of immunoreactive cells were calculated.

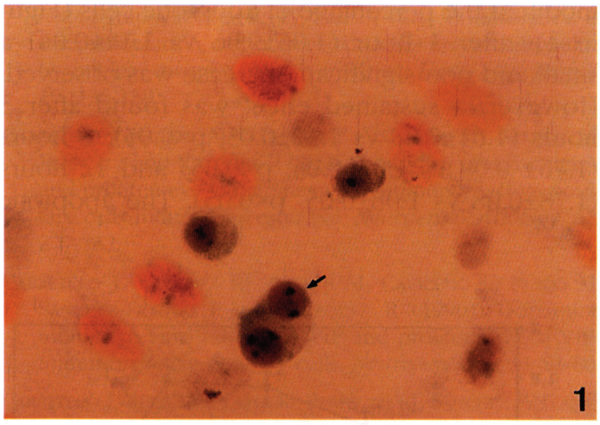
Statistical analysis. The results obtained were processed statistically and the differences observed were compared using analysis of variance, accepting $p<0.05$ as significant for the Scheffé F test. The results are expressed as arithmetic means ± standard deviation. Two pituitary cultures under similar experimental conditions were made in order to verify the reproducibility of the results (the within-assay error was less than 1.96%).

Results

Cellular proliferation. PCNA-immunoreactive nuclei were readily identified and displayed diffuse brown labelling. They were relatively evenly dispersed over the surface of the dishes, although clusters of immunopositive cells were frequently present and PCNA staining was restricted almost exclusively to the nucleus. The intensity of the reaction varied considerably: several cells showed a strongly labelled nucleus, while others displayed a weak nuclear immunoreaction. No differences in the patterns of distribution or the intensity of PCNA immunoreactivity were noted after treatment.

The percentage of PCNA-immunoreactive cells (Figure 1) was relatively high and ranged from 43 to 50% in control dishes. Immunosuppression of IL-6 induced changes in the percentages of PCNA-immunoreactive cells with respect to control dishes (Figure 2). After 1 hour of immunosuppression, a non-significant decrease was observed (43.84±1.91 vs 49.85±2.64) while very evident decreases were observed after 3 hours (34.91±2.17 vs. 50.71±2.76, $p<0.01$), 6 hours (28.41±2.11 vs. 47.56±3.02, $p<0.01$), 12 hours (36.41±1.85 vs. 46.92±2.31, $p<0.01$), but not at 24 hours (39.16±1.89 vs. 43.33±2.47, $p<0.01$).

Cellular apoptosis. BrdU-labelling appeared mainly as a brown reaction in the nuclei of cultured cells (Figure 3) except in 3.78% of the reactive cells that showed both nuclear and cytoplasmic reaction. No differences in the patterns of distribution or the intensity of BrdU immunoreactivity were noted after treatment.



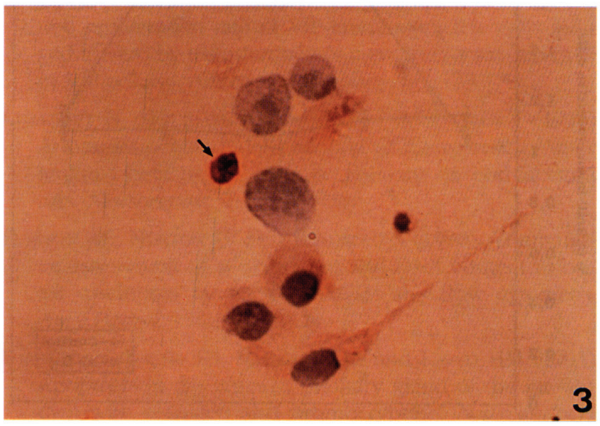


Fig. 1.– Micrograph showing immunoreactivity to PCNA (arrow) in a control culture. x450.

Fig. 3.– Micrograph showing the immunocytochemical expression of BrdU incorporated into fragmented DNA (arrow) in a culture treated with ab-IL-6. x450.

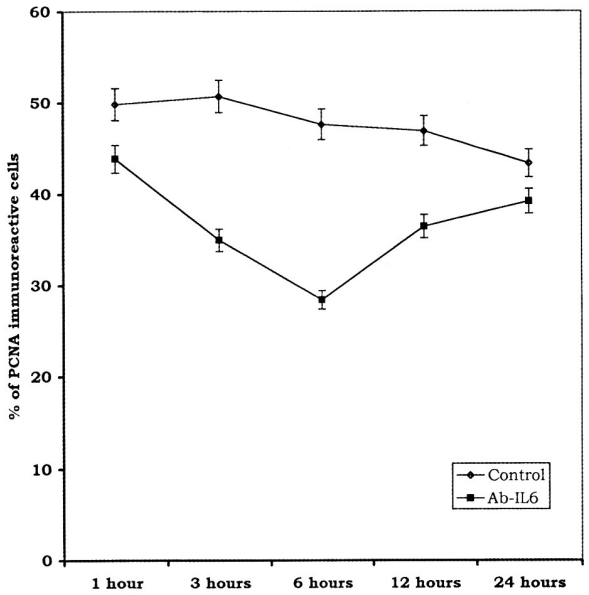


Fig. 2.– Plots of percentages of cells immunoreactive to PCNA following in vitro immunosuppression of IL-6 at the different times studied.

Control dishes did not show significant variations in the percentages of BrdU-labelled cells at the different time points analysed (Figure 4), these ranging from 1.05±0.04% to 1.40±0.06% of the total number of cells present in the cultures.

Immunosuppression of IL-6 significantly modified the percentage of apoptotic cells (Figure 4): after 1 hour (1.06±0.06 vs 1.14±0.04) a small and non-significant increase was observed. However, a sustained effect was found after 3 hours (1.61±0.08 vs 1.12±0.05, $p<0.05$), 6 hours (1.76± 0.08 vs 1.05±0.04, $p<0.05$) and 12 hours (1.78±0.08 vs 1.08±0.05, $p<0.05$). The apoptotic effects disappeared after 24 hours of immunosuppression and the values at that time were very similar to those found after 1 hour (1.13±0.09 vs 1.07±0.04).

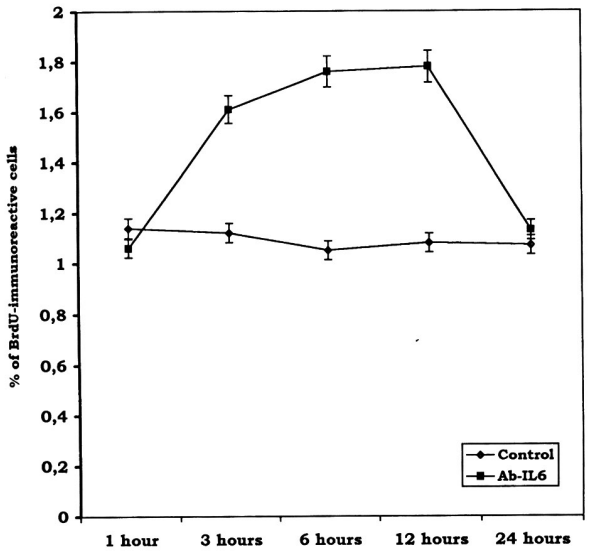


Fig. 4.– Plots of percentages of cells that incorporated BrdU in the fragmented DNA after in vitro immunosuppression of IL-6 at the different times studied.

Discussion

Because the in vitro immunosuppression of IL-6 results in an inhibition of the overall cellular proliferation of the culture, as was observed here, it is quite feasible that the in vitro effect of IL-6 on the hypophysis would be the opposite from the point of view of auto-paracrine regulation; that is cell proliferation would be stimulated.

Despite this, Artz et al. (1993) demonstrated that following the addition of IL2 or of IL-6 to hypophyseal cultures the percentage of cells incorporating tritiated thymidine decreases in a dose-dependent fashion. Such findings are apparently conflictive with the observations made here, although it should be noted that the method used by those authors to determine the percentage of proliferating cells was different from the one employed by us and that PCNA expression is up to 5-fold more sensitive than BrdU or thymidine incorporation for evaluating hypophyseal proliferation (Oishi et al., 1993). Moreover, whereas in their very elegant study those authors analysed the effect of the addition of ILs to cultures, here we analysed the effect of immunosuppression of the ILs present in the cultures. In this sense, it is difficult to compare the effects of immunosuppression versus the possible effects of an increase in IL levels in hypophyseal cultures following the addition of these cytokines.

PCNA is an auxiliary protein of DNA polymerase d, necessary for DNA replication. It is expressed in the G1 phase, reaches a maximum in the S phase, and declines again during the G2 and M phases of the cell cycle (Mathews et al., 1984; Tan et al., 1986; Prelich and Stillman, 1988). Although some discrepancies have been reported between PCNA immunoreactivity and data obtained with other cell proliferation-assessing methods, several studies have shown that the PCNA labelling index represents a valuable approach for the evaluation of cellular proliferation in human and animal tissues (Garcia et al., 1989; Dawson et al., 1990; Van Dierendock et al., 1991), including the anterior pituitary gland (Carretero et al., 1995a, 1995b, 1996, 1997).

Two types of reaction can be seen after labelling of the DNA fragmented in cellular apoptosis: at early stages, the reaction is located exclusively at the nuclear level (Gavrielli et al., 1992; Nitatori et al., 1995), while at later stages it may affect the cytoplasm, with or without nuclear reaction (Nitatori et al., 1995; Aschoff et al., 1996). In accordance with this, in the present

study we observed both types of reaction, depending on the time point analysed.

The detection of apoptotic cells is based on the most important intracellular phenomenon that occurs during apoptosis; namely, fragmentation of the DNA chains to form oligonucleotide chains of about 180 bp (Wyllie et al., 1980). By hybridization of nucleotides or their analogues to the 3' ends of the fragmented DNA and later detection of the hybridised oligonucleotides, apoptotic cells can be identified. The TUNEL method –terminal deoxynucleotidyl Transferase-mediated dUTP Nick End-Labelling– is based on this (Kressel and Groscurth, 1994; Piqueras et al., 1996; Negoescu et al., 1997), as is the ISEL method used here. Based on the in situ hybridization of BrdU to oligonucleotide probes described by Jirikowski et al. (1989, 1990), hybridization of BrdU to the 3' ends of fragmented DNA and its immunohistochemical detection using anti-BrdU monoclonal antibodies has been used to identify apoptotic cells (Aschoff et al., 1996). This method has been validated by contrasting tissues with a high cellular turnover against others with a low turnover –with and without preincubation with Dnase– obtaining excellent results (Aschoff et al., 1996). The use of this technique has a clear advantage over the TUNEL technique; i.e., the possibility of obtaining long BrdU tails of approximately 70 bp (Jirikowski et al., 1989), which form an antigen sequence that is difficult to achieve with sequences resulting from nick-translation (Aschoff et al., 1996). These advantages were observed in endocrine glands in a previous study carried out in the thyroid gland (Riesco et al., 1998).

Few studies have attempted to relate the action of ILs and cellular apoptosis. Chauvert et al. (1996) studied IL1b production in rat hypophyseal cells during the apoptosis that occurs at the end of lactation but failed to find any correlation. Our study is the first to clearly demonstrate that immunosuppression of IL-6 elicits a pronounced increase in the in vitro cellular apoptosis of the hypophysis, evidently suggesting that under normal conditions IL-6 would act as antiapoptotic agent through an auto-paracrine mechanism.

Overall, the present results show that in vitro immunosuppression of IL-6 induces a decrease in PCNA expression and an increase in BrdU incorporation into the fragmented DNA of rat hypophyseal cells. This suggests that IL-6 would act as a regulatory auto-paracrine modulator of those processes by stimulating proliferation and inhibiting apoptosis.

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