

Expression of Interleukin-6, Interleukin-6 Receptor (gp80), and the Receptor's Signal-Transducing Subunit (gp130) in Human Normal Pituitary Glands and Pituitary Adenomas

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Interleukin-6 (IL-6) is an important cytokine in cell proliferation and differentiation in several organs. It has also been reported that IL-6 plays a role in secretion or release of pituitary hormones in pituitary hormone-secreting cells and pituitary adenomas, but convincing data *in situ* have not yet been reported. In this study, we examined the participation of IL-6 in the production of pituitary hormones and the differences between human normal pituitary glands and pituitary adenomas by determination of the localization or expression of IL-6, IL-6 receptor (IL-6R, gp80), and the signal-transducing subunit (gp130) of the receptor using immunohistochemical staining and RT-PCR. IL-6 was mainly expressed in ACTH- and FSH/LH-secreting cells in normal pituitary glands, as shown by double staining. gp 80 and gp130 were coexpressed in almost all GH- and PRL-secreting cells and in approximately 30% of FSH/LH-secreting cells. RT-PCR showed that IL-6 mRNA was expressed in only one of all the pituitary adenomas examined, whereas gp 80 and gp 130 mRNAs were detected in all these pituitary adenomas. In conclusion, IL-6 was mainly expressed in ACTH- and FSH/LH-secreting cells, and the receptors were expressed in GH-, PRL- and FSH/LH-secreting cells in human normal pituitary glands. Furthermore, our data emphasized that the mechanism of IL-6 function in human pituitary adenoma cells is distinct from that in normal pituitary cells.

KEY WORDS: gp80, gp130, Human pituitary adenoma, Human pituitary gland, IL-6, Immunohistochemistry.

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Much evidence supports the existence of a neuroimmunoendocrine network (1). The function of endocrine tissues is influenced by many cytokines, which include interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), γ -interferon (IFN- γ), and transforming growth factor- β (TFG- β). These cytokines are produced not only by macrophages and lymphocytes but also by the hypothalamus or pituitary gland (2).

IL-6 is a particularly interesting cytokine: it plays a role in the acute phase response, differentiation, growth, and proliferation of various cells (3, 4). IL-6 exerts its action on target cells by acting through a receptor complex consisting of a specific IL-6-binding protein, an IL-6 receptor (IL-6R, gp80), and a signal-transducing subunit (gp130). Many studies have demonstrated that the IL-6/IL-6 complex induces the homodimerization of two gp130 molecules, leading to a number of intracellular signaling events via activation of the Jak/STAT signaling pathway (5, 6).

Although IL-6 has been shown to act as an autocrine growth factor in several tumors and is expressed by a variety of tumors (7-9), the expression of IL-6R has not been clarified in human pituitary adenomas. In the pituitary gland, many studies have demonstrated that IL-6 stimulates the release of PRL, GH, LH, and ACTH *in vitro* (10, 11). Furthermore, the bioactivity of IL-6 in cell cultures of human pituitary adenoma cells has suggested that it may influence human pituitary adenomas (12). The localization of IL-6 and gp80 has been investigated in rat and human pituitary glands by immunohistochemistry (IHC) and/or *in situ* hybridization (ISH; 13, 14); however, the localization and

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expressions have remained undetermined in the human pituitary gland or pituitary adenomas. It is known that pituitary folliculostellate (FS) cells secrete several cytokines, although the cells do not secrete any pituitary hormones. The FS cells are characterized by the starlike appearance and express S-100 protein and glial fibrillary acidic protein (15, 16). Therefore, we examined the localization of IL-6, gp80, and gp130 in human normal pituitaries and adenomas to determine their roles there.

MATERIALS AND METHODS

Patients

Three human normal pituitary glands were excised at autopsy from three patients without endocrinological abnormalities (one male, 80 years old; two females, 51 and 69 years old). The clinical and endocrinological profiles of 32 pituitary adenomas (14 males, 18 females; mean age, 51.9 years) that were excised by transsphenoidal surgery were as follows: 6 GH-secreting adenomas with symptoms of acromegaly; 6 PRL-secreting adenomas in which serum PRL levels ranged from 120 to 2560 ng/mL; 4 ACTH-secreting adenomas with typical Cushing's syndrome; 4 TSH-secreting adenomas with hyperthyroidism; and 12 nonfunctioning adenomas. All of the six nonfunctioning adenomas were positive for FSH β immunohistochemically. One human normal adrenal gland was excised at autopsy from one patient (male, 69 years old).

Immunohistochemical Staining

One human normal adrenal gland and three human normal pituitary glands were excised at autopsy within 4 hours postmortem, and the tissues were routinely embedded in paraffin after 10% formalin fixation. The tissues of the 32 pituitary adenomas were fixed in 10% formalin immediately after excision at surgery and then embedded in paraffin. After 4- μ m-thick sections were deparaffinized and rehydrated, endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 30 minutes at room temperature. The immunohistochemical studies for IL-6, gp80, and gp130 were carried out using the avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA; 17). Sections were heated for 10 minutes at 100° C in citrate buffer (pH 6.0) with a microwave oven (Energy Beam Sciences, Agawan, MA) for antigen retrieval of IL-6 (14). Sections were then incubated with each primary antibody at 4° C overnight and were incubated with normal serum for each primary antibody as negative control. The primary antibodies used in this study are listed in Table 1. After sections were reacted with biotinylated secondary antibody for 30 minutes at room temperature,

TABLE 1. List of Antibodies Used

| Antibody or (Antigen) | Source | Clonality | Dilution |
|-----------------------|--|------------------------------------|----------|
| IL-6 | R&D Systems Inc., Minneapolis, MN | monoclonal clone; 6708.111 | 1:50 |
| (IL-6) gp80 | R&D Systems Inc. Biosource, Sunnyvale, CA | monoclonal clone; B-R6 | 1:50 |
| (gp80) gp130 | Biosource Santa Cruz Biotechnology Inc., Santa Cruz, CA | polyclonal | 1:100 |
| GH | DAKO, Carpinterian, CA | polyclonal | 1:400 |
| ACTH | DAKO | monoclonal clone; 02A3 | 1:400 |
| FSH β | Immunotech, Marseille, France | monoclonal clone; 300.10.E.14.5 | 1:200 |
| PRL | NIDDK, Torrance, CA | polyclonal | 1:250 |
| TSH β | NIDDK | polyclonal | 1:3200 |
| LH β | NIDDK | polyclonal | 1:1200 |
| α -SU | NIDDK | polyclonal | 1:2000 |
| (b)S-100 protein | DAKO | polyclonal | 1:200 |

The antibodies are specific for human proteins except for anti-(b)S-100 protein antibody. (b), bovine; NIDDK, National Institute of Diabetes and Digestive and Kidney Disease.

the ABC method was carried out. In these experiments, the tissues of human normal adrenal gland were used as a positive control (17).

A double-staining technique was performed to examine the expression of IL-6, gp80, and gp130 proteins in pituitary hormone-secreting cells and S-100-positive FS cells. Briefly, the IL-6, gp80, and gp130 were stained using the ABC method, yielding a brown color with 3,3'-diaminobenzidine. The sections were then incubated in glycine-HCl buffer (pH2.2) to remove the immunocomplexes. Subsequently, the localization of the pituitary hormones or S-100 protein in the same sections was detected by an indirect method using an alkaline phosphatase-conjugated second antibody (DAKO, Carpinteria, CA), which yielded a blue color by using fast blue salt.

Two investigators independently performed cell counts to quantitate the expression of IL-6 or gp80 in pituitary hormone-secreting cells and S-100 protein-positive FS cells. For each section, three separate areas were chosen, and 150 cells per area were counted.

Adsorption Test

Adsorption tests on the human normal pituitary gland were performed to confirm the specificity for IL-6 and gp80 of each commercially obtained antibody. The antibodies for IL-6 and gp80 were monoclonal, and each epitope had been identified. For the adsorption test, each antibody was incubated with the respective antigen (Table 1) at 4° C overnight, and then the complex was centrifuged and the supernatant was passed through a 0.45- μ m filter. We could not confirm the specificity for gp130

because we could not get the information for the epitope.

Reverse-Transcription PCR Analysis

Total RNA was isolated from one normal human pituitary gland, 30 of the 32 human pituitary adenomas, and one human normal adrenal gland using TRIzol reagent, and 5 μ g of each total RNA preparation was reverse transcribed using a "Ready-To-Go," T-Primed First-Strand Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) after treatment with DNase I (Promega Biotech, Madison, WI). Polymerase chain reaction (PCR) was carried out in the reaction mixtures containing 0.4 μ M of primers; 2.5 U of r*Taq* polymerase, the buffer supplied by the manufacturer; 1 to 1.5 mM of MgCl₂; 200 μ M of deoxy-NTPs; and cDNA. The primer sequences used for PCR amplification are listed in Table 2.

After an initial denaturation at 94° C, PCR reactions were carried out using 35 cycles of 94° C for 30 seconds, the temperature for annealing for 30 seconds, and 72° C for 1 minutes, followed by incubation at 72° C for 5 minutes in a Perkin Elmer 9600 thermal cycler (PE Applied Biosystems, La Jolla, CA). PCR products were separated by gel electrophoresis on 2% agarose gel and subsequently visualized by staining with ethidium bromide.

RESULTS

Normal Pituitary Gland

Colocalization of IL-6 and Pituitary Hormones and S-100 Protein by Immunohistochemical Double Staining

The immunoreactivity of IL-6 protein was observed in the Golgi field (Fig. 1A–H). IL-6 was mainly localized in ACTH-secreting cells and in gonadotropin-secreting cells: in other words, in FSH β -secreting cells and LH β -secreting cells, α SU-positive cells (Fig. 1E–G), whereas it was scarcely observed in GH-secreting cells and PRL-secreting cells (Fig. 1A,B). IL-6 immunoreactivity was not detected at all in TSH β -secreting cells and S-100-positive FS cells (Fig. 1C,H). Table 3 summarizes the expression ratios (expressed as percentage) that were obtained by counting the IL-6-positive cells

among the anterior pituitary hormone-secreting cells and S-100 protein-positive FS cells.

Colocalization of gp80 and gp130 and Pituitary Hormones and S-100 Protein by Immunohistochemical Double Staining

First, gp80 protein (IL-6 receptor) and gp130 protein (signal-transducing subunit) were shown to be colocalized in the same cells (Fig. 2I). This result indicated that the cells expressing of gp80 were affected by IL-6 and that the signal might be transmitted to the nuclei. We then examined the localization of gp80 in anterior pituitary hormone-secreting cells and in S-100-positive FS cells. gp80 was expressed in almost all GH- and PRL-secreting cells, in 33.2% of FSH-secreting cells, and in 30.7% of α SU-positive cells (Fig. 2A,B,E,G). gp80 was expressed in only 6.08% of LH-secreting cells (Fig. 2) and was rarely expressed in TSH β -secreting cells and ACTH-secreting cells (Fig. 2C,D). In S-100-positive FS cells, no gp80 immunoreactivity was detected (Fig. 2H). The expression ratio of gp80 in pituitary hormone-secreting cells and S-100 protein-positive FS cells is shown in Table 3.

Adsorption Test

The specificity for IL-6 or gp80 was confirmed by an adsorption test using a human normal pituitary gland. Each immunoreactivity was considerably decreased by the adsorption (data not shown).

Pituitary Adenomas

Expression of IL-6, gp80, and gp130 Shown by Immunohistochemical Study

The immunoreactivities of IL-6, gp80, and gp130 proteins were generally weak in the 32 human pituitary adenomas examined. Thus, it was difficult to determine the localization of the proteins and impossible to determine the ratio of expressing cells. Thus, it required the following reverse-transcription PCR (RT-PCR) to examine the expression of the mRNAs for these proteins.

RT-PCR Analysis

The mRNA expression for IL-6, gp80, and gp130 was examined by RT-PCR in 30 human pituitary

TABLE 2. Sequence of the PCR Primers Used in This Study

| Target (Reference) | Direction | Sequence (5' to 3') | Temperature for Annealing (°C) | Product Size (bp) |
|--------------------|-----------|-----------------------------|--------------------------------|-------------------|
| IL-6 (16) | forward | TAG CCG CCC CAC ACA GAC AG | 70 | 408 |
| | reverse | GGC TGG CAT TTG TGG TTG GG | | |
| gp80 (16) | forward | CAT TGC AT TGTCT GAG GTT | 60 | 251 |
| | reverse | AGT AGT CTG TAT TGC TGA TGT | | |
| gp130 (17) | forward | CAT GCT TTG GGT GGA ATG GAC | 62 | 326 |
| | reverse | CAT CAA CAG GAA GTT GGT CCC | | |

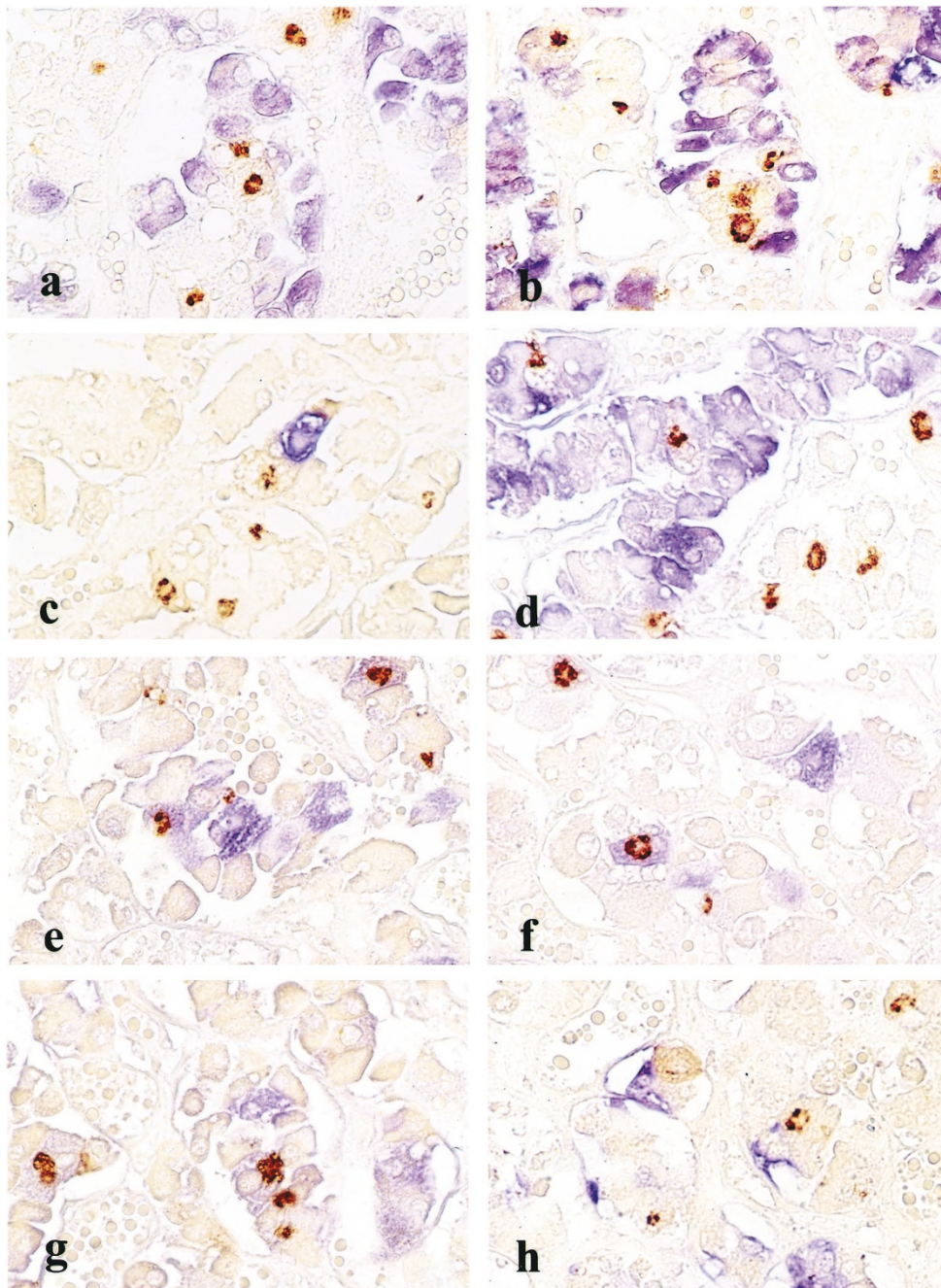


FIGURE 1. Expression of IL-6 shown by double immunohistochemical staining in human normal pituitary glands. Expression of IL-6 protein is observed in the Golgi field of anterior pituitary cells as a brown color (original magnification, 600×). The immunoreactivities of pituitary hormones and S-100 protein are shown as a blue color. IL-6 immunoreactivity was hardly expressed in (A) GH-secreting cells, (B) PRL-secreting cells, (C) TSH β -secreting cells, and (H) S-100-positive folliculostellate cells. The immunoreactivity was mainly localized (D) in ACTH-secreting cells and in gonadotropin-secreting cells (E, FSH β ; F, LH β ; G, α SU).

adenomas that were taken from the same patients analyzed by IHC. As for TSH-secreting adenomas, two cases were available for RT-PCR analysis. IL-6

mRNA was detected in only one PRL-secreting adenoma (Fig. 3A) but was not detected in the other hormone-secreting adenomas. gp80 and gp130 mR-

TABLE 3. The Expression of IL-6 or gp80 Protein in Anterior Pituitary Hormone-Secreting Cells and S-100 Protein-Positive Cells in Human Pituitary Glands

| Substance | GH | PRL | ACTH | TSH β | FSH β | LH β | α SU | S-100 |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-------|
| IL-6 | 0.96 \pm 1.27 | 0.57 \pm 1.23 | 18.4 \pm 6.8 | 0 | 30.8 \pm 3.8 | 39.0 \pm 8.2 | 41.6 \pm 13.9 | 0 |
| gp80 | 95.7 \pm 9.80 | 97.3 \pm 3.90 | 1.62 \pm 1.04 | 2.46 \pm 4.60 | 33.2 \pm 4.38 | 6.08 \pm 4.13 | 30.7 \pm 5.02 | 0 |

The data are the mean \pm SE.

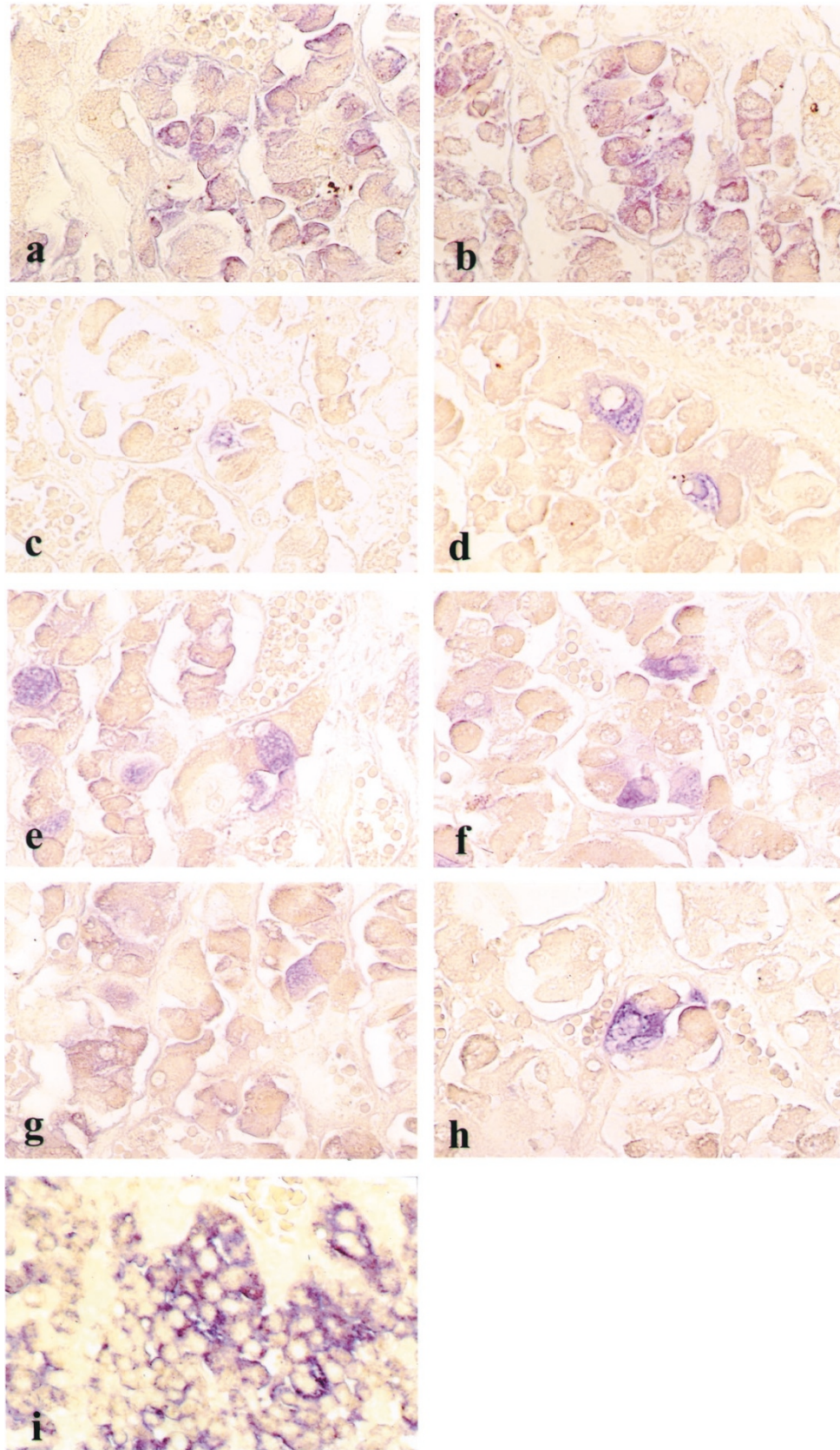


FIGURE 2. Expression of gp80 and gp130 proteins shown by double immunohistochemical staining in human normal pituitary glands. The immunoreactivities of gp80 protein (**brown**) and the other proteins (**blue**) are shown (original magnification, 600 \times). gp80 was expressed in almost all (A) GH-secreting cells and (B) PRL-secreting cells. gp80 was hardly observed (C) in TSH β -secreting cells, (D) in ACTH-secreting cells, (F) in LH-secreting cells, and (H) in S-100-positive folliculostellate cells. gp80 was occasionally expressed (E) in FSH-secreting cells and (G) in α SU-positive cells. (I) The colocalization of gp80 protein (**blue**) and gp130 protein (**brown**).

NAs were detected in all adenomas (Fig. 3B,C). PCR products of IL-6 and gp80 and gp130 were detected at 408, 251, and 326 bp, respectively.

DISCUSSION

Interleukin (IL)-6 is a multifunctional cytokine that plays roles in the stimulation, inhibition, differentiation, and regulation of cell growth. The effect of IL-6 appears to be mediated through its membrane receptor, IL-6R, and binding of IL-6 to its receptor leads to the homodimerization of gp130 and then to the activation of signal-transducing tyrosine kinases (5, 6, 18). IL-6 has been reported as an autocrine and/or paracrine growth factor in several tumors (3, 4, 7–9) and is known to induce the secretion or release of GH, PRL, ACTH, and LH/FSH in rat pituitary glands *in vivo* and/or *in vitro* (10, 11). Several groups have examined the expression of IL-6 protein and mRNA and gp80mRNA in human normal pituitary glands and pituitary adenomas (13, 14, 19, 20). However, the localization of IL-6, gp80, and gp130 in human normal pituitary glands and their expressions in human pituitary adenomas have remained unclear. In this study, we examined the participation of IL-6 in the production of pituitary hormones and the differences in the pathway of this production between human normal pituitary glands and pituitary adenomas using immunohistochemical staining and RT-PCR.

The results of double staining in normal pituitary glands indicated that IL-6 was mainly localized in ACTH and gonadotropin-secreting cells and that the

immunoreactivity was clearly detected in the Golgi regions. In rat normal pituitary glands, it has been reported that the major site of IL-6 expression is in FS cells. In contrast, in human normal pituitary glands, it has been reported that IL-6 was hardly found *in situ* in FS cells (21). These latter data are consistent with our results that IL-6 was not detected in FS cells. However, in human normal pituitary glands, IL-6 had previously been examined *in situ*, but its expression pattern had not been established. Therefore, our results about the localization of IL-6 are very important data that were obtained using an anti-human IL-6 antibody that is highly specific for IL-6 protein.

Furthermore, double staining for gp80 and pituitary hormones or gp80 and S-100 protein was performed to understand whether the cells are affected by IL-6 through an autocrine and/or paracrine pathway. The coexpression of both gp80 and gp130 proteins in the same cells suggested that IL-6 functions in pituitary hormone-secreting cells. In particular, our results revealed that gp80 was expressed in the vast majorities of GH- and PRL-secreting cells. It has previously been shown that GH, PRL, and LH are released in response to stimulation by IL-6 in cell perfusion experiments on rat pituitary glands (10). Thus, our data indicated that IL-6 directly influences the production of GH and PRL in human pituitary glands. IL-6 was expressed in 30.8% and 41.6% of FSH and α SU-positive cells, respectively, but only in 6.08% of LH-positive cells. We have not elucidated how IL-6 acts on FSH- and LH-secreting cells; however, our results suggest that IL-6 might play roles in these cells through autocrine/paracrine pathways. gp80 was detected in few ACTH-secreting cells (0.62%). IL-6 has been reported to be a potent activator of the hypothalamic-pituitary-adrenal (HPA) axis *in vivo* and *in vitro* (22, 23). Recently, it has been clarified that the release of ACTH induced by IL-6 is mediated through CRH secreted from the hypothalamus. The secretion of CRH is increased by 5-HT (5-hydroxytryptamine, serotonin), whose release is stimulated by IL-6. Concerning our findings of gp80 expression in ACTH-secreting cells, it is reasonable to suggest that the release of ACTH induced by IL-6 is mediated through CRH (23).

On the other hand, in human pituitary adenomas, the immunoreactivities of IL-6, gp80, and gp130 protein were very weak. The results of RT-PCR demonstrated that IL-6 mRNA was not expressed in any of the adenomas examined except for in one PRL-secreting adenoma, whereas in contrast, gp80 and gp130 were expressed immunohistochemically in all of the pituitary adenomas. In the PRL-secreting adenoma that was positive for IL-6 mRNA, IL-6 immunoreactivity was detected in infiltrating lymphoid cells (data not shown). These results indicated that human pituitary adenomas do not produce IL-6 but do produce gp80 and gp130. Some investigators have studied IL-6 or gp80 in

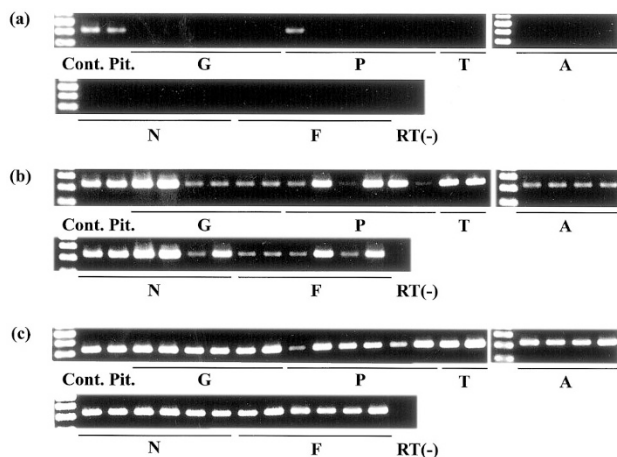


FIGURE 3. Reverse-transcription PCR analysis for IL-6, gp80, and gp130 in human pituitary adenomas. (A) IL-6 mRNA was detected in only one PRL-secreting adenoma at 408 bp. (B) gp80 mRNA and (C) gp130 mRNA were detected in all adenomas. PCR products of gp80 and gp130 were detected at 251 and 326 bp, respectively. Cont., human adrenal gland as a positive control; Pit, human normal pituitary gland; G, GH-secreting adenoma; P, PRL-secreting adenoma; A, ACTH-secreting adenoma; N, nonfunctioning adenoma; F, FSH β -positive nonfunctioning adenoma; T, TSH-secreting adenoma; RT(-), non-reverse-transcribed total RNA.

human pituitary adenomas and reported that IL-6 is produced by cultured human pituitary adenoma cells, and they also suggested that some of the IL-6 released may have originated from contaminating macrophages, fibroblasts, or endothelial cells (12). IL-6 mRNA and protein have also been identified within human pituitary adenomas by *in situ* hybridization and IHC (12, 13), but our findings on IL-6 expression differed from those results. However, our immunohistochemical results were confirmed by highly sensitive RT-PCR, and the disparity in IL-6 production by human pituitary adenoma cells is thought that the report for IL-6 in cultured human pituitary adenoma cells includes two abilities of contamination of macrophages and the difference for sensitivity in detection of IL-6. IL-6 bioactivity was analyzed by the proliferation of IL-6-dependent mouse hybridoma cell line B9 (12), but we analyzed the production by IHC in tissues. The data suggested that pituitary adenomas could be affected by IL-6 produced in lymphoid cells and other cells or in normal pituitary cells surrounding the adenomas. Further, our data suggested that the possibilities include down-regulation of IL-6, gp80, and gp130 during neoplastic transformation of the pituitary cells. The mechanism by which the expressions were weak in human pituitary adenomas remained for future investigation.

Our conclusions are as follows: our results suggested that IL-6 may function in GH and PRL-secreting cells through paracrine and endocrine pathways and that IL-6 may function in FSH-secreting cells through an autocrine pathway. Furthermore, our results supported the proposal that the secretion of ACTH is increased by CRH, which is induced by IL-6, in human normal pituitary glands. Furthermore, our data emphasized that the mechanism of IL-6 function in human pituitary adenoma cells is different from that in human normal pituitary cells and that the adenoma cells could be under regulation of IL-6 for the extrapituitary cell source.

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