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Research Article

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Human and Murine Pituitary Expression of Leukemia Inhibitory Factor

Novel Intrapituitary Regulation of Adrenocorticotropin Hormone Synthesis and Secretion

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Abstract

Leukemia inhibitory factor (LIF) gene expression was detected in human fetal pituitary tissue by expression of LIF mRNA transcripts, protein immunocytochemistry, and immunoelectron microscopy. Fetal LIF immunoreactivity colocalized with 30% of ACTH-expressing cells, ~ 20% of somatotrophs, and ~ 15% of non-hormone-expressing cells. LIF was also strongly expressed in normal adult pituitary and in four growth hormone-producing and two ACTH-producing adenomas, but not in eight nonfunctioning pituitary tumors. Culture of fetal cells expressing surface LIF-binding sites demonstrated predominance of in vitro ACTH secretion as compared with other pituitary hormones. In AtT-20 murine cells, LIF (ED_{50} 10 pM) stimulated basal proopiomelanocortin mRNA levels by 40% and corticotropin-releasing hormone-induced ACTH secretion (two- to threefold), as did oncostatin M (ED_{50} 30 pM), a related peptide. ACTH responses were not further enhanced by both cytokines together, which is consistent with their shared receptor. Anti-LIF antiserum neutralized basal and LIF-induced ACTH secretion, suggesting autocrine regulation of ACTH by LIF. The results show that human pituitary cells express the LIF gene and LIF-binding sites, predominantly in corticotrophs. Pituitary LIF expression and LIF regulation of proopiomelanocortin and ACTH reflect an intrapituitary role for LIF in modulating early embryonic determination of specific human pituitary cells and as a paracrine or autocrine regulator of mature ACTH. (*J. Clin. Invest.* 1995. 95:1288-1298.) Key words: pituitary adenoma • Cushing's disease • acromegaly • pituitary cytokine

Introduction

Mechanisms controlling embryologic development of fetal pituitary tissue may provide important insights into the differentiation of pituitary function. Cells of the anterior pituitary express highly differentiated trophic hormone gene products, and their cytogenesis from a common pituitary stem cell involves complex nuclear and membrane signaling systems, resulting in specific cell types secreting either growth hormone (GH),[1] prolactin (PRL), adrenocorticotropin (ACTH), thyroid-stimulating hormone (TSH), and gonadotropin (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) hormones (1). Each of these trophic hormones is under stimulatory or inhibitory mediation by unique hypothalamic hormones that bind to specific pituitary cell surface receptors (2).

Specific pituitary cell types are also regulated by intrapituitary cytokines and growth factors that mediate cell growth or local regulation of angiogenesis. The pituitary is an abundant source of several growth factors, including basic fibroblast growth factor, which modulates PRL and TSH release in vitro (3, 4). Epidermal growth factor modulates pituitary secretion in vitro (5-7), and transforming growth factor-α is expressed by pituitary lactotrophs (8). Insulin-like growth factor I is expressed and regulated in somatotrophs and has been recognized as a mediator of GH synthesis (9, 10). Interleukins 1 and 6 have also been detected in rat and human pituitary tissue (11), and these cytokines have been shown to regulate the corticotropin-releasing hormone (CRH)-ACTH axis in vitro (12) and in vivo (13).

Since growth and differentiation of hormone-secreting and non-hormone-secreting pituitary cells may be mediated by paracrine regulators of angiogenesis and endothelial cell growth (14), we investigated whether the physiological development of the human pituitary is associated with expression of leukemia inhibitory factor (LIF). LIF, originally isolated as an inhibitor of mouse M1 myeloid leukemia cells (15), stimulates proliferation of hematopoietic progenitor cells, maintains the developmental potential of embryonic stem cells by suppressing differentiation, modulates several differentiated cell functions, and promotes differentiation of cholinergic neurons (16). LIF and oncostatin M (OSM) share 25% amino acid homology (17, 18), activate a common gp130 receptor subunit (19), and are produced by a variety of cell types, including bone marrow stromal cells, thymic epithelial cells, fibroblasts, and a number of malignancies (17).

LIF has been shown to be secreted by cultured bovine pituitary follicular cells and to regulate bovine vascular systems (20). We therefore investigated the expression of LIF in human fetal and adult pituitary and tested the action of LIF on pituitary function. The results show that the LIF gene is expressed in the developing human fetal pituitary and in both normal and tumorous adult pituitary tissue. Using AtT-20 murine corticotroph cells, LIF and OSM were also shown to stimulate proopio-

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1. *Abbreviations used in this paper:* CRH, corticotropin-releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; LIF, leukemia inhibitory factor; LIF-R, leukemia inhibitory factor receptor; OSM, oncostatin M; POMC, proopiomelanocortin; PRL, prolactin; TSH, thyroid-stimulating hormone.

melanocortin (POMC) mRNA and basal and CRH-induced ACTH secretion. These LIF actions as well as endogenous ACTH secretion are blocked by immunoneutralizing anti-LIF antiserum. The results indicate an intrapituitary autocrine or paracrine mediation of the CRH–ACTH axis by LIF.

Methods

Human fetal pituitary tissue. The use of human fetal tissue followed guidelines outlined by the National Advisory Board on Ethics in Reproduction (21). Tissues were derived from third party referrals for therapeutic termination to an independent facility with no direct or indirect participation of the investigators in the termination decision. Written informed consent was subsequently obtained by the facility for anonymous distribution of aseptic tissue specimens.

RNA extraction and ribonuclease protection assay. Fetal tissue or AtT-20 cells were used for RNA extraction and subsequent ribonuclease protection assay (22). Tissues were harvested within several minutes and kept at −70°C. Total tissue RNA was extracted after homogenization by RNA STAT-60 (TEL-TEST' B'', Inc., Friendswood, TX). RNA concentrations were estimated by ultraviolet spectrophotometry at 260 nm and 260/280 ratio. Aliquots of RNA samples were electrophoresed through 1% agarose/6.9% formaldehyde gels, and RNA integrity was confirmed by visualization with ethidium bromide.

The BamHI-HindIII fragment of the human LIF cDNA clone or the EcoRI-Xbal fragment of the murine LIF cDNA (23) (kindly provided by Dr. Tracy Willson, The Walter Eliza Hall Institute of Medical Research, Melbourne, Australia) was inserted into a vector (pBluescript II SK +, Stratagene, La Jolla, CA) and used as templates for generating the RNA probes. pTR1-β-actin, human or murine antisense (Ambion, Austin, TX), was used as an internal standard. The resulting constructs were linearized with NotI (human), HindIII (mucine), gel purified, and subsequently used to generate a LIF antisense probe. The probe labeling was performed using [α-^{32}P]UTP (Du Pont–New England Nuclear, Boston, MA) and in vitro transcription kits (Maxiscript T3/T7, Ambion), followed by gel purification of the riboprobe in a 5% urea/acrylamide gel. T7 RNA polymerase was used for both LIF and β-actin (human), and T3 RNA polymerase was used for both LIF and β-actin (murine). The labeled probes were eluted overnight at 37°C in a gel elution buffer containing 2 M ammonium acetate, 1% SDS, and 25 μg/ml transfer RNA, with shaking. The probes for LIF and β-actin were each precipitated with 20 μg of human tissue RNA extract, 100% ethanol, and 5 M ammonium acetate. The assay was performed using a ribonuclease protection assay (RPA 2 kit, Ambion), following the manufacturer's instructions. Reaction products were redissolved in 20 μl of 80% (vol/vol) formamide hybridization buffer. Each test sample contained 100,000 cpm, and incubations were for 21 h at 42°C. 20 μg of total CEM (human T cell line) RNA was used as a tissue specificity control. Nonhybridized RNA was digested with RNase for 1 h at 37°C. After redissolving in 8 μl of gel loading buffer, denaturation was performed at 95°C for 3 min, and then a 5% urea/acrylamide gel (1500 V) was used for a 3-h separation. The hybridization was visualized by exposure to x-ray film (Fuji, Kanagawa, Japan) for 48 h at −80°C. The level of the β-actin expression was used as an internal standard to correct for RNA loading. As negative and positive human tissue controls, fetal liver, spleen, intestine, and heart muscle RNAs were also tested. Murine embryonic fibroblasts (STO cells) were used as positive murine cell controls.

Primary fetal pituitary cell culture and cell separation. Specimens were harvested in low glucose DME supplemented with 0.3% BSA, 2 mM glutamine, and antibiotics. The pituitary was dissected under a stereomicroscope, and one-half of the tissue was washed carefully, minced with a sterile surgical scalpel, and enzymically dissociated using 0.035% collagenase and 0.01% hyaluronidase (both from Sigma Chemical Co., St. Louis, MO). Cell suspensions were filtered and resuspended in low glucose DME supplemented with 2.5% FCS, 2 mM glutamine, and antibiotics after washing. The remainder of the specimen was used for cell sorting or immunohistochemistry.

For primary cultures, $\sim 10^4$ cells were seeded in multiwells and incubations were continued for up to 24 h, after which the medium was collected and stored at −20°C for later hormone measurement by commercial radioimmunoassay (Nichols Institute, Reference Laboratories, San Juan Capistrano, CA). Cells collected after FACS sorting (see following section) were similarly incubated in vitro. These included cell populations that were positive for both CD surface antigen and biotinylated LIF receptor (LIF-R), as well as a population of LIF-R–negative cells.

Immunofluorescence staining and cell sorting. Fresh single-cell suspensions were double stained using both direct and indirect immunofluorescence procedures (24). 10^6 cells were first incubated with conjugated phycoerythrin (PE) or FITC-conjugated mixed murine mAbs to CD2, 3, 4, 8, 14, and 19, which are reactive with lymphocytes (T, natural killer, and B), monocytes, and macrophages (25, 26) at various concentrations, respectively. Mouse IgG1- and IgG2-conjugated PE (1 μg/ml) were used as negative controls. For indirect staining, biotinylated LIF (10 μg/ml) (Fluorokine TM, R & D Systems, Minneapolis, MN) (27) was used to detect cell surface LIF binding (LIF-R), and avidin–FITC or avidin–PE (10 μg/ml) was used for the subsequent step for test samples and for the initial step for negative controls. Cells were incubated at 4°C for 30–60 min for both direct and indirect stainings and washed twice between antibody additions. Centrifugation was performed at 1,500 rpm for 10 min for every step (Beckman GS-6R Centrifuge; Beckman Instruments, Inc., Palo Alto, CA). PBS and RDF1 buffer (Fluorokine TM) at 4°C were used as the suspension medium. Specimens were analyzed on a fluorescence-activated cell sorter (FACStar, Becton Dickinson Immunocytometry Systems, San Jose, CA) using the 488-nm line of an argon laser. FITC and PE signals were measured simultaneously and were electronically compensated to reduce spectral overlap. FACS analysis of AtT-20 cells (4×10^6/ml) was performed using biotinylated LIF and avidin–fluorescein. As controls, cells were exposed to avidin–fluorescein alone, or to biotinylated LIF in the presence of excess unconjugated LIF. Data were analyzed using computer software (Lysis II, Becton Dickinson Immunocytometry Systems). Fluorescence was calibrated against reference standards (Flow Cytometry Standards Corp., Puerto Rico).

Immunohistochemistry of fetal pituitary tissue. Double immunostaining was performed on formalin-fixed, paraffin-embedded sections (28) using a 1:20 dilution of goat anti–human LIF antiserum (1 mg/ml IgG, R & D Systems) in conjunction with one of the following: rabbit anti–human GH (DAKO Corp., Carpinteria, CA), rabbit anti–human PRL (DAKO Corp.), rabbit anti–human ACTH (DAKO Corp.) (all at 1:500 dilution), rabbit anti–human TSH-β (lot No. AFP 55741789; 1:5,000 dilution), rabbit anti–LH-β (AFP 55951889; 1:2,000 dilution), or rabbit anti–FSH-β (AFP 20102688) from the National Hormone and Pituitary Program, NIDDKD, NICHD, and the USDA, diluted 1:1,000. After the primary antisera, slides were incubated sequentially with swine anti–goat IgG antibody peroxidase conjugate (Boehringer Mannheim, Indianapolis, IN) and alkaline phosphatase–conjugated goat anti–rabbit immunoglobulins (DAKO), both at 1: 40 dilution. Diaminobenzidene peroxidase substrate (Sigma Immunochemicals) and the fast red substrate system (DAKO) were used as chromogens, and slides were counterstained with methyl green. For specificity controls, serial sections were incubated with anti–human LIF antiserum preadsorbed with excess human LIF (500 ng/ml; R & D Systems), or isotype-specific non–cross-reacting immunoglobulin (mouse anti–goat IgG; Sigma Immunochemicals).

Immunoelectron microscopy. Ultrastructural localization was performed on cryostat sections using methods previously described (29). Sections were placed in polylysine paraformaldehyde fixative without air drying. After rinsing with PBS, slides were stained with goat anti–human LIF antiserum. Prior to the diaminobenzidene reaction, slides were postfixed in 3% gluteraldehyde in 0.1 M cacodylate buffer for 30 min. Sections were then postfixed in 1% osmium tetroxide and embed-

ded in Eponate 12 resin (Ted Pella Inc., Redding, CA) for ultramicrotomy.

Immunohistochemistry of normal adult pituitary and pituitary adenomas. Staining for LIF was performed on paraffin sections as previously described (30). Tissue from four normal pituitaries was obtained at autopsy from patients dying of multiple myeloma, myocardial infarction, non-Hodgkin's lymphoma, and primary pulmonary arterial hypertension. Sections from pituitary neoplasms were obtained from the surgical pathology files of Cedars–Sinai Medical Center and included eight nonfunctioning adenomas, one prolactinoma, two ACTH-producing adenomas, and four GH-producing adenomas. Sections were deparaffinized and incubated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity before immunostaining using methods previously described.

Culture of AtT-20 cells. AtT-20 cells were routinely maintained in Ham's F-10 medium (Gibco BRL, Grand Island, NY) supplemented with 15% horse serum and 2.5% FCS (31). Before test incubations, cells were serum starved for 16 h. Cells were transferred to fresh serum-free DME at a density of 50,000–100,000 cells per ml in microfuge tubes and recombinant human LIF (R & D Systems), OSM (R & D Systems), and CRH (ovine tyr-corticotropin–releasing hormone; Sigma Chemical Co.) were applied at appropriate concentrations in 10 μl of serum-free medium. An equal volume of medium alone was applied to control tubes. After test incubations, cells were pelleted by centrifugation, and the supernatant was stored (−20°C) for later ACTH assay using a two-site kit (Nichols Institute). All test incubations were performed in triplicate and assayed in duplicate. Values are expressed as mean±SEM, and statistical significance was assessed by unpaired *t* test.

Northern analysis of AtT-20 mRNA. AtT-20 cells ($\sim 7 \times 10^6$ cells per group) were preincubated for 16 h in serum-free DME and then incubated for an additional 24 h in fresh serum-free DME with or without added treatments. Total cell RNA was extracted using TRIzol Total RNA Isolation Reagent (Gibco, Gaithersburg, MD). Cell pellets obtained by centrifugation were lysed in TRIzol Reagent, and total RNA was isolated by adding chloroform and centrifuging (32). Isolated RNA was precipitated using isopropanol. The RNA pellet was washed using 75% ethanol, denatured in 2.2 M formaldehyde plus 44 mM Mops, pH 7.0, containing 50% deionized formamide, 10 mM sodium acetate, 1 mM EDTA, and 0.25 mM ethidium bromide at 55°C for 15 min, separated by electrophoresis on a 1.2% agarose gel containing 0.37 M formaldehyde and 44 mM Mops at 100 V for 3 h, and transferred to nylon membrane in transfer buffer containing 3 M sodium chlorate and 0.3 M sodium citrate, pH 7.0, at room temperature overnight. The membrane was baked at 80°C for 2 h washed in distilled deionized water, prehybridized in 10 ml of hybridization solution (QuikHyb Rapid, Stratagene) at 68°C for 30 min, and hybridized with $\sim 10^6$ cpm ^{32}P-labeled murine POMC cDNA at 68°C overnight. Prehybridization and hybridization were performed in a ISS Hybridization oven (Enprotech, Natick, MA). After hybridization, the membrane was washed twice at room temperature for 15 min each in 2× SSC, 0.1% SDS and twice in 0.1× SSC, 0.1% SDS, followed by air drying and autoradiography.

Murine POMC cDNA, kindly provided by Dr. Malcolm J. Low (Portland, OR) (33), was propagated in DH5-α–competent cells (Gibco) and separated using Qiagen-tip 2500 (QIAGEN Inc., Chatsworth, CA). A 0.6-kb fragment, spanning the 3′ half of exon 3 of the murine POMC gene, was digested with NcoI and XbaI and isolated from the pGEM-7 (Promega Corp., Madison, WI) vector by electrophoresis in a 1.2% agarose gel. The isolated cDNA was extracted by a DNA gel extraction kit (QIAEX, QIAGEN Inc.) and labeled with [α-^{32}P]CTP and DNA polymerase using a random primer labeling kit (Prime-IT II, Stratagene).

Results

Human fetal and murine LIF mRNA expression. RNA was freshly extracted from fetal tissue for measurement of LIF mRNA expression (Fig. 1). RNase protection assay of fetal pituitary RNA revealed a protected LIF mRNA transcript of 400 bp. The RNA obtained from CEM cells, a human T cell leukemia line known to express LIF, was used as a positive tissue control. Pituitary tissues obtained at 14 and 16 wk gestation expressed protected mRNA hybrids that were similar in size to those observed for spleen and intestine obtained at 16 wk gestation. No LIF mRNA expression was detected in liver and diaphragm obtained at 16 wk. In contrast, when AtT-20 cell RNA was subjected to an RNase protection assay with a murine full-length cRNA, protected transcripts of 644, 621, and 400 bp were detected (Fig. 2). These bands were similar in size in the murine embryonic fibroblast control cells.

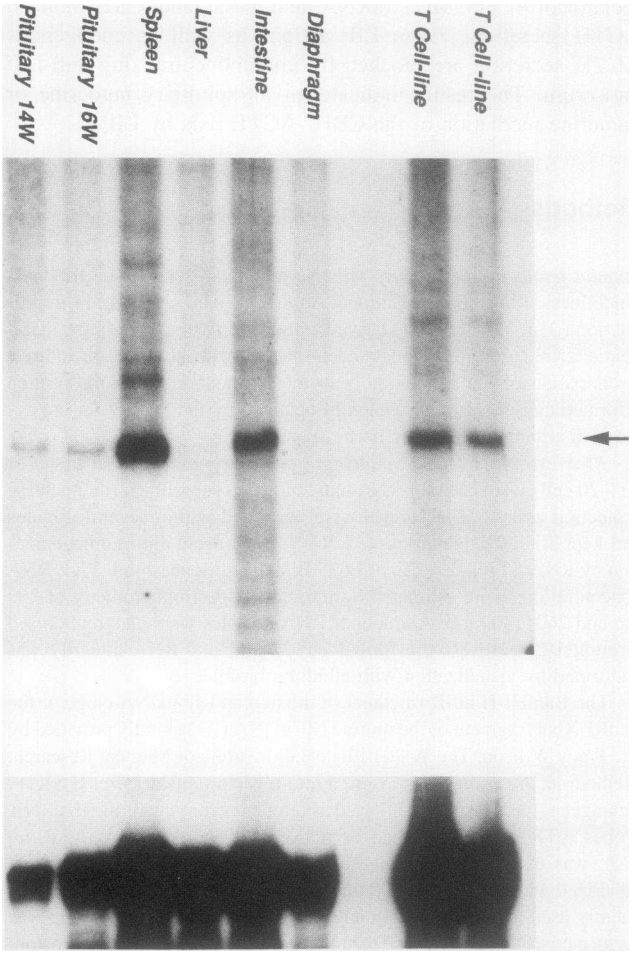

Figure 1. RNase protection assay of human fetal tissues derived at 14 (14W) or 16 (16W) wk. RNA extracts (20 μg per lane) were hybridized with ^{32}P-labeled LIF cRNA ($\sim 10^5$ cpm) and subjected to RNase digestion. Protected fragments (arrow) were 400 bp in size. The bottom panel depicts β-actin mRNA protected transcripts, which served as internal standards.

Immunohistochemistry of fetal pituitary cells. Cells revealed staining for both LIF and LIF-binding sites (LIF-R). One-third of the ACTH-positive cells coexpressed LIF and LIF-R (Table I), and ~ 20% costained for GH. As expected, a greater percentage of cells stained for GH than PRL, and LIF coexpression was more prevalent in GH- than in PRL-producing cells. 10–16% of cells staining for TSH, PRL, gonadotrophins, or α subunit, respectively, also expressed LIF immunoreactivity. 17% of cells negative for trophic hormones stained for LIF.

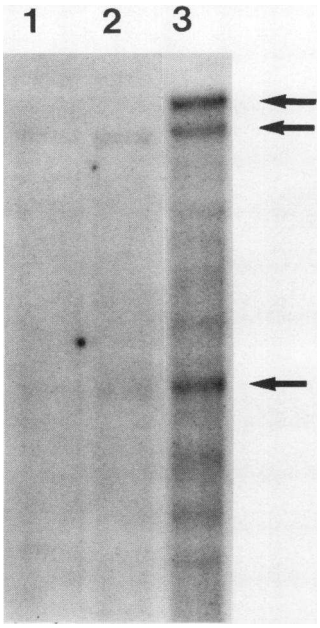

Figure 2. RNase protection assay of murine AtT-20 cells. RNA extracts (20 μg per lanes *1* and *2*) were hybridized with ^{32}P-labeled murine LIF cRNA ($\sim 10^5$ cpm). Protected fragments (*arrows*) are 644, 621, and 400 bp in size. Lane *3*, murine embryonic fibroblast (STO cell) RNA.

Control slides (serial sections incubated with preabsorbed antisera or nonimmune sera) were negative for LIF or their respective trophic hormones.

Immunoelectron microscopy of fetal pituitary cells. After immunostaining with anti-LIF antiserum, finely granular electron-dense reaction product was localized to the cytoplasm of isolated single pituitary cells. Staining was restricted to the region of secretory granules and concentrated near the cell membrane (Fig. 3). Other cells, including those with secretory granules, were nonimmunoreactive. Negative control sections with substitution of the primary antiserum were unstained.

Population of pituitary cells determined by FACS. To determine the expression of LIF surface binding in individual fetal pituitary cells, dispersed cells were subjected to FACS sorting. A representative result of sorted human fetal cells obtained at 19 wk gestation is depicted in Fig. 4 *A*. The majority of the fetal pituitary cells were both LIF-R and CD series negative. Cells binding LIF (LIF-R positive), cells that express CD series surface antigens (mix of CD2, 3, 4, 8, 14, and 19), and cells stained for both LIF and CD were separated. The population of cells staining exclusively for the CD markers was undetectable at 16, 19, and 27 wk, with a maximal value of only 0.6% detected at 24 wk. Cells expressing LIF-R but negative for CD series surface antigens were considered specific pituitary sources of LIF binding and were barely detectable at 16 wk, highest at 17 wk ($\sim$ 38% of all cells), and present only in $\sim$ 2% of cells at 27 wk, reflecting single stained pituicytes expressing LIF-R. Specific LIF binding to AtT-20 cells was also demonstrated by fluorescence flow cytometry, and binding was inhibited by 50% in the presence of excess unconjugated (free) LIF (Fig. 4 *B*).

Hormone secretion by sorted human fetal pituitary cells. Pituitary hormones were measured in media from cultures of unsorted cells obtained at 19, 20, and 27 wk gestation, and in vitro hormone levels were also measured after cell sorting and subsequent culture (Table II). Hormone concentrations increased in a time-dependent manner in the presorted cell cultures. ACTH, FSH, LH, and GH were all detected in presorted cells derived at 19 wk gestation, whereas PRL and TSH were detected, albeit at very low levels, at 20 wk. After sorting, cultures of LIF-R–positive cells, presumably reflecting cultured human pituicytes containing LIF-binding sites, secreted significant levels of ACTH. In contrast, the other anterior pituitary hormones were undetectable in these cultures derived from LIF-R–positive cells at 19, 20, and 27 wk gestation.

LIF immunostaining in normal adult pituitaries and pituitary adenomas. To determine the adult expression of pituitary LIF, normal pituitary tissue sections as well as 15 pituitary adenomas were immunostained for LIF (Table III). Four normal pituitaries stained strongly for LIF (Table III), whereas variable staining occurred in pituitary adenomas. Strong (two cases) and intermediate (two cases) staining was present in GH-producing adenomas, and intermediate staining was also observed in two ACTH-producing adenomas. Negligible LIF staining (trace to weak) was present in eight nonfunctioning adenomas and one prolactinoma.

LIF action on cultured fetal pituitary monolayers. To study the effects of exogenous LIF on human fetal ACTH secretion, cells (16 wk gestation) were seeded, and after 24 h, incubations continued in serum-free defined medium with added LIF. ACTH levels were almost doubled by LIF (50 ng/ml), whereas no significant change in concentrations of the other pituitary hormones was detected. Because of the difficulty in obtaining larger numbers of human fetal tissue specimens, as well as the technical problems inherent in primary cultures of hormone-secreting human fetal tissue, subsequent in vitro experiments were performed using the AtT-20 murine corticotroph cell line.

LIF regulation of POMC gene expression and ACTH secretion. As AtT-20 cells also expressed LIF and contained LIF-binding sites, they were considered a relevant model for studying in vitro pituitary LIF action on POMC gene expression.

AtT-20 cells were exposed to LIF in serum-free conditions for up to 24 h, and POMC mRNA levels were measured. Basal POMC expression was high, and mRNA levels were readily measurable when only 8 μg of total RNA was subjected to

Table I. Percentage of Human Pituitary Fetal Cells Immunoreactive for Adenohypophyseal Trophic Hormones and for LIF or LIF-R

	ACTH	GH	TSH-β	PRL	FSH-β	α subunit	LH-β	No hormone costaining
LIF (%)	33 (32–33)	21 (17–26)	16 (13–18)	13 (11–16)	10 (6–14)	11 (9–13)	9 (6–11)	17 (14–20)
LIF-R (%)	35 (31–40)	21 (19–25)	13 (10–16)	15 (11–18)	8 (8–11)	9 (7–10)	8 (6–11)	15 (12–18)

Values were obtained by examining 20 different microscopic fields in each of three serial slides obtained from the same specimen. Percentages reflect the cells staining for LIF or LIF-R among the immunopositive trophic cells. Values are means (and ranges) obtained from three different pituitary specimens at 20 wk gestation.

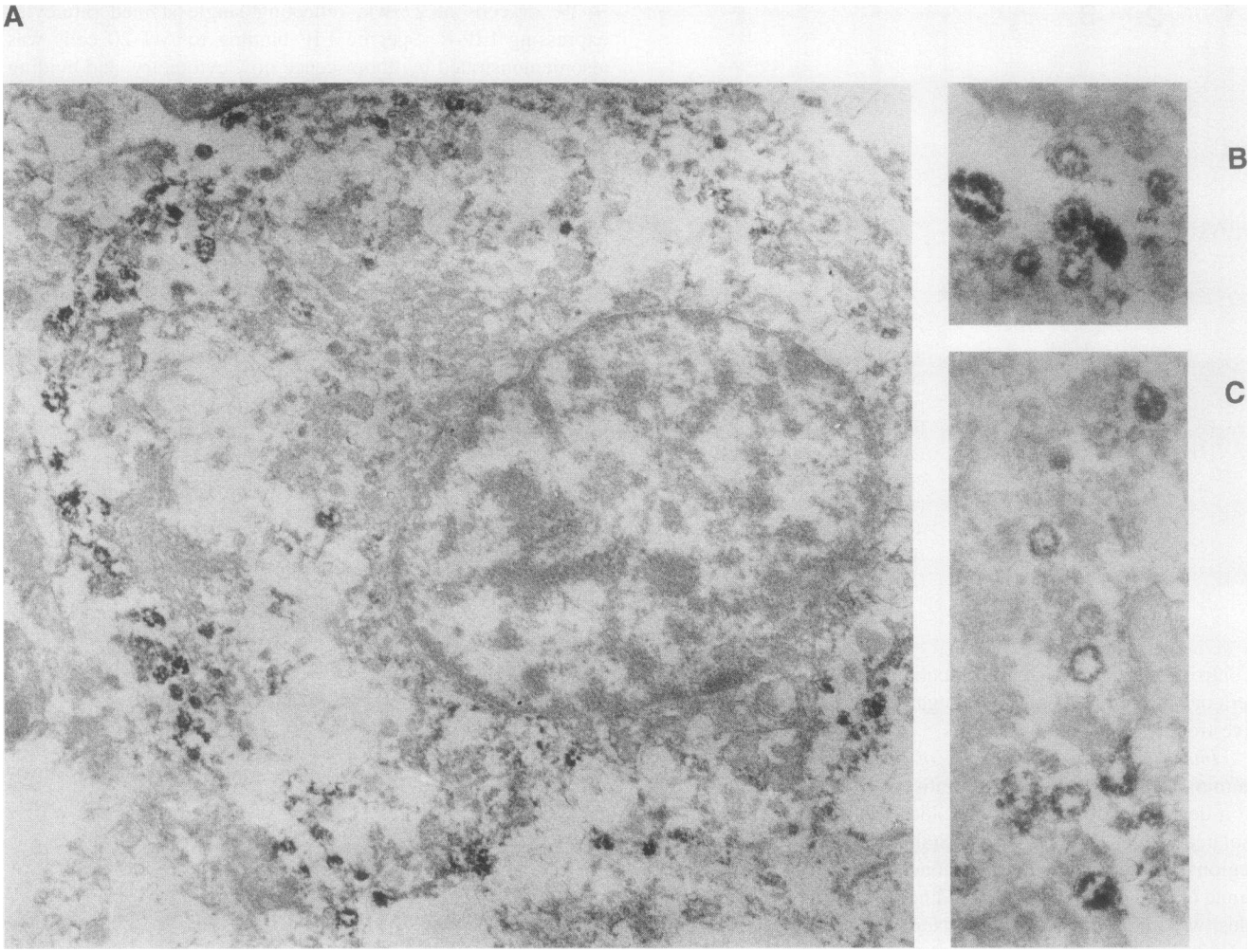

Figure 3. Immunoelectron microscopy of human fetal pituitary cells (20 wk gestation) stained with anti-human LIF antiserum. The electron-dense reaction product is localized to cytoplasmic granules concentrated near the cell membrane. (*A*) Low power electron micrograph. ×9,500. (*B* and *C*) Higher power electron micrographs of LIF-positive cytoplasmic granules. ×17,500.

hybridization (Fig. 5). Both LIF and OSM induced the levels of POMC mRNA after an 8-h incubation (Fig. 6). LIF (100 or 1,000 pM) induced the levels of POMC transcripts by over 50%, and 1 nM OSM similarly stimulated POMC mRNA.

ACTH secretion by AtT-20 cells was stimulated by both LIF and OSM (Fig. 7). This effect was detectable within 6 h of exposure to the cytokine and persisted for at least 72 h. The ED_{50} values for LIF and OSM were 10 and 30 pM, respectively. Significant stimulation of ACTH was observed with 1 pM LIF ($P = 0.027$) and 10 pM OSM ($P = 0.045$), and the maximal stimulation, achieved at a concentration of 300 pM at 24 h, was 60–80% above basal ACTH secretion for both cytokines. When the cytokines were applied in combination, at concentrations that, individually, elicited maximal stimulation of ACTH secretion (1 nM), no further stimulation of ACTH secretion was observed (Fig. 8).

CRH, as expected, was a potent stimulator of ACTH secretion, increasing secretion up to threefold during 24-h incubations. Coincubation of AtT-20 cells with CRH and either LIF or OSM resulted in a significant ($P < 0.001$) potentiation of ACTH secretion: compared with cells exposed to CRH alone, the addition of LIF or OSM resulted in a two- to threefold enhancement of the ACTH response to CRH (Fig. 8). Exposure to CRH together with maximal doses of LIF and OSM together did not significantly increase ACTH concentrations, compared with CRH and LIF or CRH and OSM, respectively.

To confirm the relevance of endogenously produced LIF in regulation of ACTH secretion, experiments were performed in the presence of specific anti-LIF antiserum or nonimmune sera. As shown in Fig. 9, the addition of immunoneutralizing anti-LIF antiserum to cell cultures suppressed basal ACTH secretion, in the absence of exogenously added LIF. Using either anti-human LIF (1:100 or 1:60) or anti-murine LIF (1:60) antiserum, dose-dependent suppression of ACTH secretion during 24 h was observed as compared with ACTH secretion in the presence of nonimmune goat serum (1:60). Anti-LIF antiserum (1:60) also attenuated the induction of ACTH by exogenous LIF (1 nM) during 24-h incubations.

Discussion

These results show that LIF is expressed in human fetal and adult pituitary tissue. In the human fetal pituitary, LIF expression was confirmed by light microscopic immunostaining, by

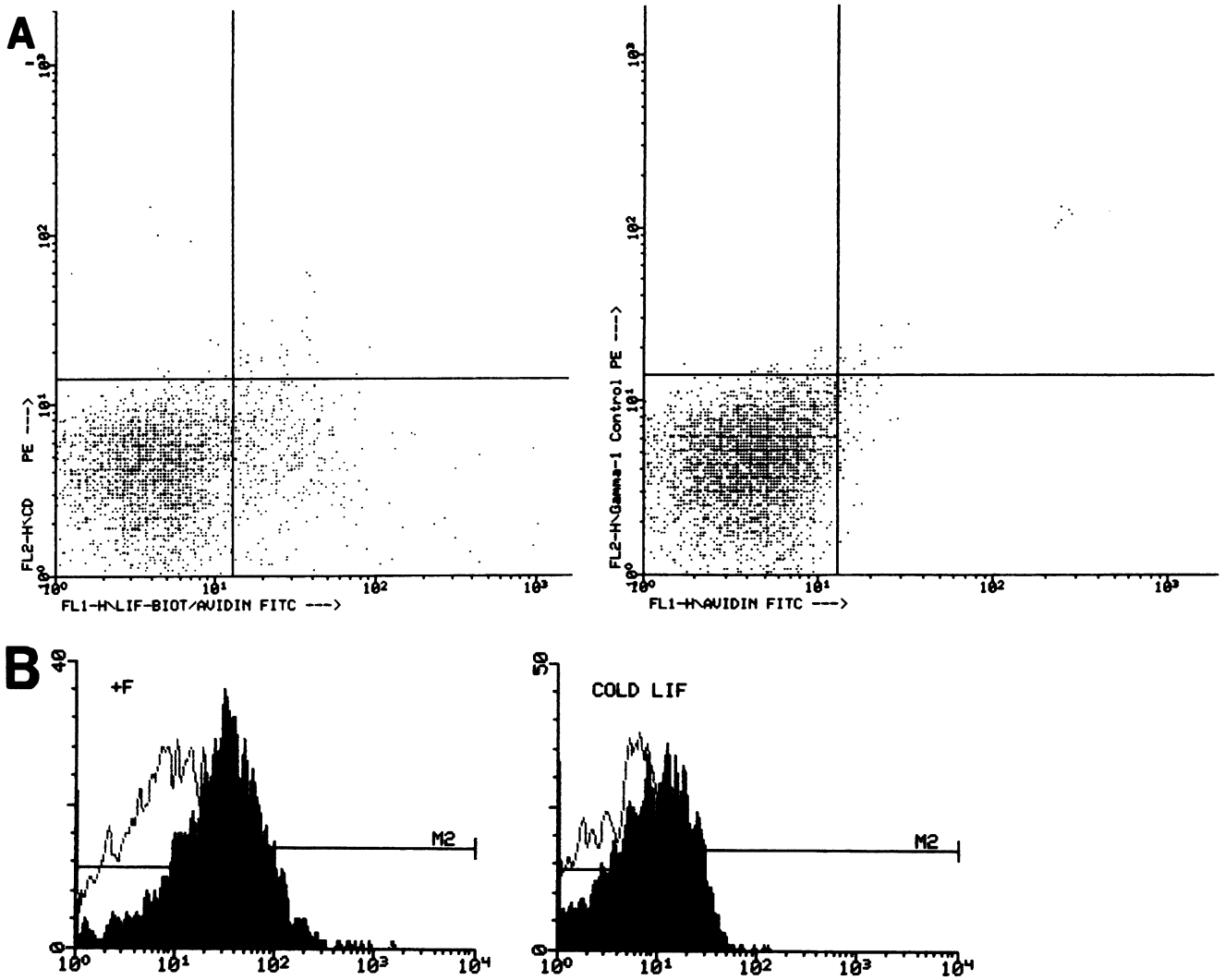

Figure 4. (*A*) FACS-sorted fetal pituitary cells obtained at 19 wk. Cells were exposed to PE and FITC followed by antibodies against the CD series of antigens and biotinylated LIF. The coordinate shows LIF-R and the Y coordinate represents the CD series of cell surface antigens. Cell population in the lower right quadrant (*left*) subtracted from the corresponding control lower right quadrant (*right*) represents the population (12.03%) of cells expressing surface LIF-binding sites. (*B*) FACS sorting of AtT-20 cells exposed to biotinylated LIF with or without excess unconjugated LIF. Nonspecific LIF binding (~ 8%) was subtracted from total cell binding. The percentage of cells binding LIF decreased from 41.16 (*left*) to 21.4% (*right*) in the presence of unconjugated LIF.

immunoelectron microscopy, and by a specific RNase protection assay. Pituitary cell surface LIF-binding sites were demonstrated by FACS and by immunostaining in human fetal pituitary tissue and in AtT-20 cells. LIF appears to colocalize mainly in fetal ACTH-secreting cells, and exogenous LIF induces basal and CRH-induced ACTH secretion and POMC mRNA levels in AtT-20 cells. Anti-LIF antiserum attenuates LIF induction of ACTH and inhibits endogenous ACTH secretion, indicating intrapituitary regulation of endogenous POMC gene expression and ACTH secretion by LIF. As immunoreactive LIF is also present in human adult normal and tumorous corticotroph and somatotroph cells, LIF may also play a role as a neuroimmune modulator of adult pituitary cell function.

Adenohypophyseal cells appear to arise from an early stem cell that subsequently undergoes differentiation and compartmentalized commitment to express the specific anterior pituitary trophic hormones (1). Cell-specific transcription factors for GH, PRL, and TSH have been described (1). Although murine models for cell-specific development of POMC expression (33) have recently been developed, little information is available on the ontogeny of the human corticotroph cell. In the rat, expression of ACTH precedes that of other trophic hormones (1). In the human, immunoreactive ACTH is detected in Rathke's pouch by week 5 (34, 35), before development of a mature human hypophyseal–portal vascular system, which occurs between the 8th and 14th week. At 14 wk, the fetal corticotroph is sensitive to CRH stimulation (36). These observations imply that factors other than hypothalamic CRH are involved in determining the cell-specific expression of the early fetal corticotroph. Dramatically increased intensity of ACTH-immunoreactive pituitary cells occurs through the end of the second trimester. In the rat, increased corticotroph proliferation occurs in response to CRH (37), and the mature adult pituitary cell population consists of 10–20% corticotrophs (35). Human fetal pitu-

Table II. In Vitro Human Fetal Pituitary Hormone Concentration

	ACTH *pg/ml*	GH *ng/ml*	TSH *mIU/liter*	PRL *ng/ml*	LH *mIU/ml*	FSH *mIU/ml*
19 wk presorted	192 (5)*	22.7 (<0.6)	0.5 (<0.2)	0.9 (<0.4)	13 (<2)	5 (<0.5)
Nonspecific Sorted	44	4.2	0.4	ND	ND	ND
LIF-R^+/CD^-	24	0.7	0.4	ND	ND	ND
20 wk presorted	626	89	1.2	1.5	40	3.9
Nonspecific Sorted	77	2.2	0.3	ND	5	ND
LIF-R^+/CD^-	68	ND	ND	ND	2	ND
27 wk presorted	5,370	3,160	181	27	75	22.1
Nonspecific Sorted	2,220	354	21	5	13	3.5
LIF-R^+/CD^-	21	0.7	0.7	ND	ND	ND

Each value represents mean hormone concentration in triplicate pituitary samples cultured for 24 h. Aliquots of cell suspension were cultured either before (presorted) or after FACS sorting (see Fig. 4). Nonspecific FACS-sorted cells include all pituitary cells that were CD positive and LIF-R positive or negative. LIF-R^+/CD^- cells include pituicytes that were LIF-R positive. After sorting, cell numbers ranged from 10^3 to 10^4 per well. * Values in parentheses indicate the blank value assayed in cell-free medium. ND, not detectable.

itary LIF expression was examined only at 16 wk, by which time the CRH axis would be intact. Pituitary-derived LIF may therefore play a role in the paracrine regulation of corticotroph cell function. Studies at earlier gestational ages are required to determine whether LIF and/or LIF-R expression precedes that of ACTH. Although cytokine production by non–ACTH-secreting cells may signal for ACTH regulation, enriched rat pituitary corticotroph cells do in fact respond appropriately to CRH in the absence of other pituitary cell types (38).

Table III. Adult Pituitary LIF Immunostaining

	Clinical diagnosis	LIF staining grade
1	CNF	+
2	CNF	Trace
3	CNF	+
4	CNF	+
5	CNF	Trace
6	CNF	Trace
7	CNF	+
8	CNF	+
9	Acromegaly	+++
10	Acromegaly	+++
11	Acromegaly	++
12	Acromegaly	++
13	Cushing	++
14	Cushing	++
15	Prolactinoma	+
16	Normal pituitary	+++
17	Normal pituitary	+++
18	Normal pituitary	+++
19	Normal pituitary	+++

Specimens for histochemistry include surgically derived pituitary adenomas and autopsy-derived normal human pituitary tissue. CNF, clinically nonfunctioning tumor; (+) weak staining; (++) moderate staining; (+++) strong staining.

Murine LIF transcripts of different sizes have been observed (39, 40). Using a 600-bp radiolabeled human cRNA excluding the first exon, the 400-bp protected human pituitary LIF mRNA detected in fetal pituitary tissue is consistent with divergent splicing of the human LIF gene, as has been suggested for the murine LIF gene (41). In murine embryonic stem cells, alternative LIF transcripts are independently expressed and may give rise to either a diffusible or matrix-associated form of the protein (40). The three protected mRNA bands detected in AtT-20 cells are similar in size to the previously reported three murine embryonic cell LIF transcripts (40). Whether the 400-bp protected transcript detected in human fetal tissue reflects a similar functional splicing distinction is unclear and requires further study.

FACS sorting revealed that both human fetal pituitary cells and AtT-20 corticotroph cells express surface sites that bind biotinylated LIF. This binding was displaceable by excess unlabeled LIF ligand. The use of antibodies against CD series antigens enabled clear distinction of the relatively small population of macrophage or monocytic cells that may have been infiltrating the pituitary, thus allowing isolation of a population of endogenous human fetal pituicytes expressing LIF surface binding markers. Furthermore, using biotinylated LIF, immunoreactive LIF-binding sites were also detected by immunochemistry in both hormone-secreting and non–hormone-secreting fetal pituitary cells. ACTH-secreting cells appear to account for the majority of secretory fetal pituitary cells cultured after sorting for LIF-R, and using costaining techniques, ACTH coexpression was detected in about one-third of LIF- and LIF-R–immunoreactive cells. Nevertheless, the relative sensitivities of the pituitary trophic hormone assays may have precluded earlier detection of non-ACTH hormones, especially GH, in the LIF-R–positive pituitary cultures.

Several cytokines are known to interact with the hypothalamo–pituitary axis, modulating the output of pituitary hormones (11), and several of these are expressed in the rat and human pituitary. In human pituitary tumors, IL-2, IL-2 receptor, and IL-6 have been shown to be expressed by either immunostaining, in situ hybridization, or direct in vitro assay (42–

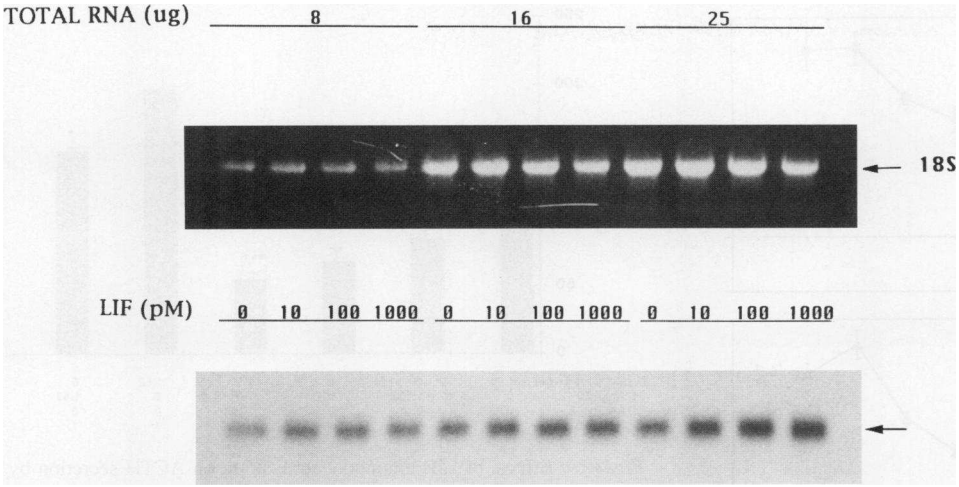

Figure 5. POMC mRNA regulation by LIF. AtT-20 cells were treated as indicated for 24 h. Extracted RNA (8, 16, or 25 μg per lane) was subjected to Northern hybridization with radiolabeled POMC cDNA ($\sim 10^7$ cpm). Gels were visualized and scanned as described in Methods. The top panel depicts an ethidium bromide RNA loading gel. Arrows indicate 18S RNA and 1 kb.

45). Although IL-2 expression has been described in Cushing's tumors (43), the functional tumor cell type distribution of IL-6 is presently unclear, as some reports indicate that the cytokine is confined to ACTH- and GH-producing adenomas (44), whereas others describe its expression in all functional tumor cell types, including clinically nonfunctioning tumors (42, 45).

Fetal pituitary LIF expression occurred predominantly in functional hormone-expressing cells. Although corticotroph and somatotroph cells appeared to account for about half of all LIF-positive cells, ~ 15% of non–hormone-secreting cells were also immunopositive for LIF and LIF-R. These nonfunctional cells may in fact be human counterparts of the rodent folliculo–stellate cells, which also express IL-6 (46). Interestingly, of the adult pituitary specimens, clinically nonfunctioning pituitary tumors did not express LIF, unlike the functional GH- and ACTH-secreting tumors. LIF immunoreactivity was also strongly expressed in normal human pituitary tissue. This observation should be interpreted with caution, as these autopsy-derived specimens may reflect generalized overexpression of brain cytokines during the preterminal period. Ideally, surgically derived normal pituitary tissue would be required to confirm this observation; however, these specimens are not readily available, as there are few nontumorous indications for surgical hypophysectomy. The adult distribution of pituitary LIF immunoreactivity is therefore similar to the human fetal pituitary cell type distribution, in which corticotrophs and somatotrophs were associated predominantly with LIF coexpression.

IL-1α, IL-1β, IL-2, and IL-6, as well as IL-6– and IL-2–binding sites, have been detected in rat pituitary tissue (47, 48). IL-1α and IL-1β are induced in response to systemic injection of the bacterial LPS (49, 50) and may be involved in the endocrine stress response. CRH release is induced by IL-1 and IL-6 (12); although IL-1 and IL-6 also stimulate rat and murine ACTH release and POMC mRNA levels (51, 52), the evidence for a direct pituitary role of IL-1 has been conflicting (12).

LIF and OSM are two members of a recently defined family of structurally and functionally related cytokines that include IL-6, IL-11, and ciliary neurotrophic factor (17). Although LIF and its receptor differ in structure from IL-6 and its receptor, they share a common gp130 receptor subunit (19). Signaling by the LIF receptor follows a 1:1 heterodimerization of the gp130 subunit with the high affinity LIF receptor β subunit, resulting in a high affinity LIF-binding site (53). In contrast, IL-6 signaling involves homodimerization of gp130 with a low affinity IL-6–binding protein (54). OSM and LIF share a number of structural and functional similarities (19), and OSM binds to the high affinity (but not the low affinity) LIF receptor, probably accounting for the broad overlap between the biological effects of the two cytokines. It is therefore not surprising that the two ligands have similar actions on ACTH expression. ACTH stimulation observed during exposures to LIF and OSM is consistent with an effect on gene expression as well as on peptide release. The ED_{50} values for ACTH stimulation by LIF and OSM are in the expected physiologic range for high affinity LIF receptor binding and are comparable to those observed for other biological actions of these peptides (16).

The absence of an additive effect on ACTH when LIF and OSM were applied together to AtT-20 cells is consistent with what is known of the two cytokines and their receptors and suggests that the two are acting via the same pathway. The synergistic interaction between LIF or OSM and CRH on ACTH secretion indicates that different pathways are involved in con-

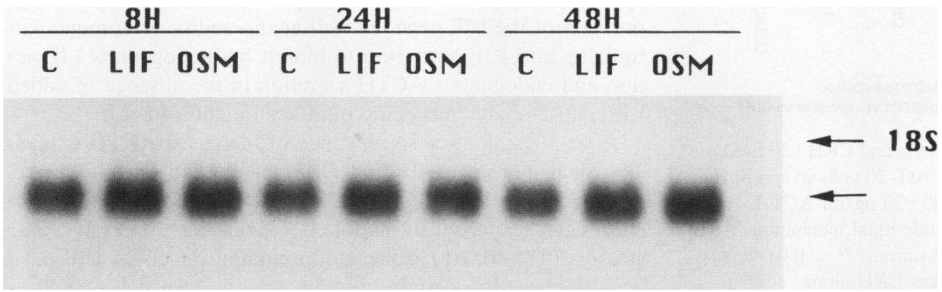

Figure 6. POMC mRNA regulation by LIF and OSM. AtT-20 cells were treated as indicated for 8–24 h. RNA was extracted and hybridized as for Fig. 5. The bottom arrow indicates 1 kb.

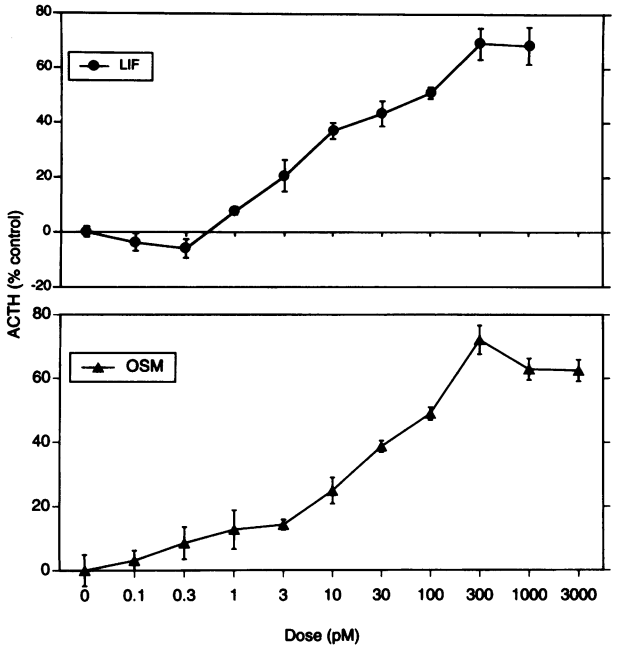

Figure 7. Dose response of LIF or OSM effects on ACTH secretion by AtT-20 cells during a 24-h incubation. Cells were cultured in serum-free defined medium with the indicated doses of either cytokine. Control cells secreted 7,230±140 pg/ml ACTH during 24 h. Each value represents mean±SEM of triplicate wells from a representative experiment performed four times. LIF induction of ACTH was significant (P = .027) at 1 pM; OSM induction of ACTH was significant (P = 0.045) at 10 pM.

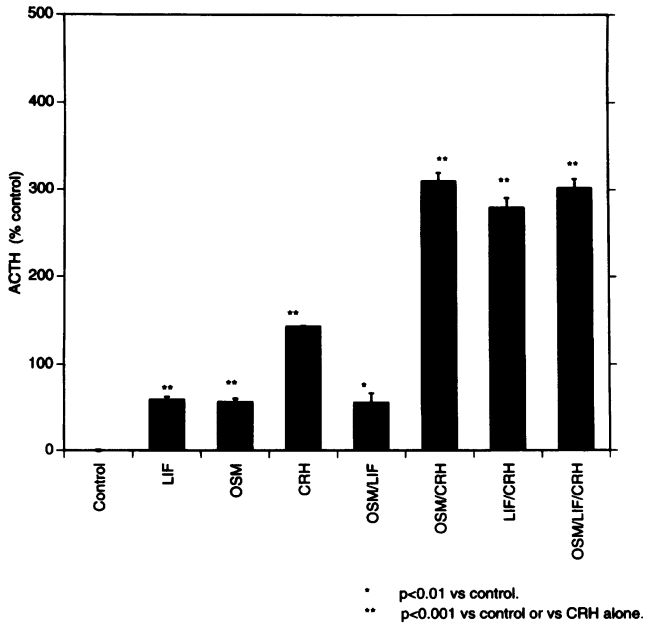

Figure 8. Effects of LIF (1 nM), OSM (1 nM), and CRH (20 nM) on ACTH secretion during a 24-h incubation of AtT-20 cells in serum-free defined medium. Control cells secreted 6,600±20 pg/ml ACTH. Each bar represents mean±SEM of three to four individual incubations from a representative experiment performed three times. *P < 0.01 versus control, **P < 0.001 versus control or versus CRH alone.

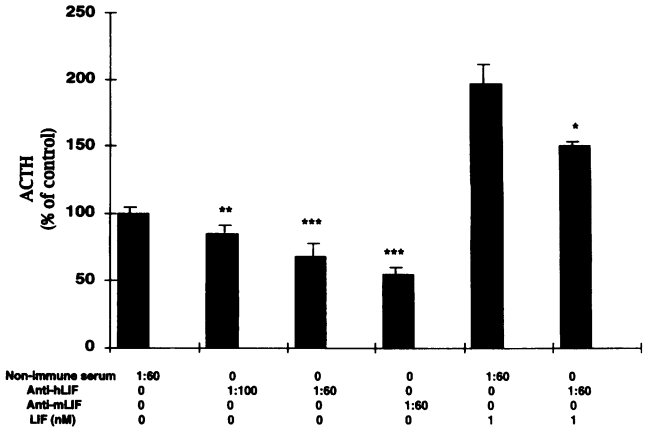

Figure 9. Effects of LIF immunoneutralization on ACTH secretion by AtT-20 cells during 24 h. Cells were incubated in the presence of nonimmune serum or anti-human or anti-murine LIF antiserum at the indicated titers. LIF (1 nM) was added as indicated. *P < 0.05 versus LIF, **P < 0.05 versus control, ***P < 0.01 versus control.

veying the two sets of positive signals for POMC. The CRH receptor is a transmembrane, Gs-coupled, adenylyl cyclase–activating molecule (55). Several non-cAMP intracellular mediators of LIF and OSM include the Jak/Tyk kinases, p91, and mitogen-activated protein kinases (56). However, the mechanism by which LIF/OSM stimulate POMC gene expression and ACTH secretion and the possible involvement of the above intracellular mediators and their interaction with cAMP-dependent pathways have not been established. LIF and OSM do not potentiate the cAMP response to CRH in AtT-20 cells (data not shown), despite their potentiating CRH effects on ACTH. Interestingly, IL-1 has recently been shown to stimulate ACTH in AtT-20 cells by activating protein kinase A via an undefined, non–cAMP-dependent mechanism (57).

LIF inhibits growth of murine leukemia cells (15), inhibits embryonal stem cells (16), and blocks aortic endothelial cell proliferation (20). OSM also possesses growth-inhibiting activity (17). Recently, IL-6 and IL-2 have been shown to inhibit [^{3}H]thymidine incorporation into adult rat pituitary cells (58). These cytokines may therefore regulate pituitary mitotic activity in addition to their effects on hormone secretion.

Although LIF, OSM, and to a lesser extent IL-6 and granulocyte colony-stimulating factor share some similar functions, their divergent amino acid sequences suggest that they also possess unique nonredundant functions (18). For example, inhibition of stem cell differentiation (54) and regulation of cholinergic neurons (59) appear unique to LIF. It appears that although LIF and OSM may act via the same receptor, whereas IL-6 acts through its own distinct receptor, the effects of this cytokine family on ACTH overlap. Nevertheless, pituitary expression of the LIF gene, as well as the ability of immunoneutralizing anti-LIF antiserum to inhibit both exogenous LIF action and endogenous ACTH secretion in the absence of added LIF, substantiates a specific pituitary function for LIF.

The physiological significance of increased ACTH expression in response to LIF and OSM has yet to be established. These two cytokines, however, may be mediators of the neuroendocrine response to endotoxic shock or other systemic stresses (13, 60, 61). Elevated circulating levels of LIF have been described in certain inflammatory disease states, such as

giant cell arteritis (60) and septic shock (61), and it has been proposed that circulating or locally produced cytokines may influence pituitary function during systemic stress (13). Pituitary cytokine interactions may occur independently of CRH (62), and autocrine or paracrine modulation of ACTH secretion independent of changes in CRH may also occur during systemic stress (63). Nevertheless, the factors controlling LIF expression and action within the pituitary are not yet known. Overexpression of murine LIF causes a fatal early cachexia. These ill mice show evidence of multiple organ damage as well as adrenal atrophy (64). Whether the loss of adrenal lipid is due primarily to LIF or to the effects of severe cachexia is difficult to determine. LIF knockout mice exhibit retarded growth and suppression of hematopoietic stem cells, and females characteristically are unable to implant a fertilized ovum successfully (65), suggesting that uterine LIF facilitates blastocyst implantation. Endometrial and pituitary LIF expression and function may therefore be important effectors of the immune–endocrine interface. Furthermore, LIF has been suggested to regulate development of pituitary angiogenesis (20) and is here shown to be expressed in both fetal and adenomatous pituitary tissue. It may therefore play a role in pituitary embryogenesis as well as tumorigenesis.

Evidence for an intrapituitary paracrine or autocrine regulatory mechanism for corticotroph development and function presented in these studies suggests the presence of a novel autocrine or paracrine LIF–ACTH regulation that may be a determinant of differentiated corticotroph cell development and function.

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