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Reduced Expression of the Cytokine Transducer gp130 Inhibits Hormone Secretion, Cell Growth, and Tumor Development of Pituitary Lactosomatotrophic GH3 Cells

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Two of the most potent cytokines that regulate anterior pituitary cell function are leukemia inhibitory factor and IL-6. These and others like IL-11 and ciliary neurotrophic factor are referred to as the gp130 cytokines because they share the gp130 glycoprotein as a common receptor initial signal transducer. We and others have shown that gp130 cytokines and their receptors are expressed and functional in normal and tumoral anterior pituitary cells. To study the role of gp130 cytokines in tumorigenic process, we generated gp130 cDNA gp130 sense and gp130 antisense (gp130-AS) transfected stable clones derived from lactosomatotroph GH3 cells. We examined hormone secretion and cell proliferation of these clones as well as their tumorigenic properties in athymic nude mice. Although gp130-AS clones, which have low gp130 levels and impaired signal transducer and activator of transcription 3 activity and suppressor of cytokine signaling-3 expression, showed reduced proliferation and hormone secretion (GH and prolactin) in response to gp130 cytokines, they had a normal response to gp130-independent stimuli. Moreover, gp130-AS clones showed a severely impaired *in vivo* tumor development. In contrast, the overexpressing gp130 clones (gp130 sense) showed no differences, compared with cells transfected with control vector. Thus, the present study provides new evidence supporting a link between gp130 and pituitary abnormal growth. (*Endocrinology* 144: 693–700, 2003)

FOR IL-6 AND OTHER cytokines that belong to the IL-6 cytokine family, their receptor cytoplasmic domains are not required for cytokine-mediated signal transduction, which is mediated by another membrane glycoprotein-denominated gp130 (1, 2). The gp130 glycoprotein alone does not bind IL-6 or other cytokine of its family, and the binding of these cytokines (i.e. IL-6) triggers the association of their α-subunits with gp130 (*i.e.* IL-6Rα chain with gp130). All those cytokines that make use of the gp130 chain belong to the gp130 cytokine family, which is composed by IL-6, leukemia inhibitory factor (LIF), IL-11, oncostatin M, ciliary neurotrophic factor (CNTF), cardiotropin-1, cardiotrophin-like related cytokine, and stimulating neurotrophin-1/B cell-stimulating factor-3 cell (3). Gp130 mainly activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway and also induces a family of cytokine-inducible signaling inhibitors including among others suppressor of cytokine signaling-3 (SOCS-3; Ref. 4).

Considering that gp130 is ubiquitously expressed, the responsiveness of a given cell type to gp130 cytokines is therefore mainly determined by expression of the respective specific α-subunits and the cytokines themselves. This takes place in the pituitary gland in which specific receptors for IL-6 (5), LIF (6), IL-11 (7, 8), and CNTF (8) have been described, providing the basis for the regulatory role of these cytokines in both hormone secretion and cell growth (9). In addition, the JAK/STAT/SOCS-3 pathway has also been described in pituitary cells (10, 11).

It has been demonstrated that these cytokines regulate hormone secretion in pituitary cell lines, normal anterior pituitary cells, and human tumor pituitary cells. For example, IL-6 stimulates the release of prolactin (PRL), GH, and LH (12, 13). IL-6 and LIF are also powerful stimuli for ACTH secretion by corticotrophs *in vitro* and *in vivo* in different species (13–16). Induced expression of proopiomelanocortin mRNA by IL-6 (17) as well as the enhanced ACTH secretion and proopiomelanocortin expression by IL-11 was reported (7). Similarly, IL-11 and CNTF regulate the secretion of GH and PRL in lactosomatotrophic cells (GH3 cell line) (8), human pituitary adenomas (18), and vascular endothelial growth factor (VEGF) release in folliculostellate (FS) cells (TtT/GF cell line) (8). The role of gp130 has not, however, been studied so far.

Besides the effects of gp130 cytokines on the secretion of pituitary hormones, gp130 cytokines also have influence on pituitary cell growth. Thus, we previously demonstrated that IL-6 regulates pituitary cell growth (19). Intriguingly, IL-6 stimulates DNA synthesis and cell number of tumor GH3 cells, yet at the same cytokine concentrations, it inhibits the

Abbreviations: C/EBP, CCAAT/enhancer-binding protein; CNTF, ciliary neurotrophic factor; FCS, fetal calf serum; FS, folliculostellate; gp130-AS, gp130 antisense; gp 130-S, gp130 sense; JAK, Janus kinase; LIF, leukemia inhibitory factor; LUC, luciferase; PRL, prolactin; RSV, Rous sarcoma virus; SOCS-3, suppressor of cytokine signaling-3; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.

growth of normal rat pituitary cells (19). IL-6 has been also reported to stimulate the growth of TtT/GF cells (20) as well as the proliferation of the MtT/E rat tumor pituitary cell line (21). Accordingly, TtT/GF cells, which also express IL-6, has been shown to stimulate the somatotrophic tumor cells (MtT/S) pituitary tumor growth in nude mice (22). In different tumors (ACTH-, PRL-, GH-secreting and nonfunctioning adenomas), IL-6 has been shown to have either inhibitory or stimulatory effects. However, the type of response does not seem to be associated with tumor type or size. By using blocking antibodies in a functional assay to study intrapituitary action, it has been demonstrated that endogenous IL-6 regulates the expression of c-fos in a similar manner (23). CNTF and IL-11 stimulate the proliferation of FS and GH3 cells (8). The fact that, in addition, these cytokines (IL-6, LIF, IL-11, and CNTF) are expressed in normal and tumoral pituitary cells (9, 13–16) suggests that they have important paracrine/autocrine actions in the pituitary gland, which could be mediated by gp130.

Based on this evidence, we generated stable GH3 clones expressing different gp130 levels to study the role of the cytokine common transducer gp130 glycoprotein in the regulation of lactosomatotrophic function and growth.

Materials and Methods

Cell culture and stimulation

Materials and reagents, except where stated, were obtained from Seromed (Berlin, Germany), Invitrogen (Carlsbad, CA), Life Technologies, Inc. (Frederick, MD), Sigma, (St. Louis, MO), and Nunc (Wiesbaden, Germany). Recombinant rat CNTF (R&D Systems Inc., Minneapolis, MN) and TRH (Bachem, Bubendorf, Switzerland) were used.

GH3, a rat lactosomatotrophic pituitary tumor cell line obtained from the American Type Culture Collection (Manassas, VA) (19), and GH3 gp130 clones, generated as indicated below, were used. They were cultured in DMEM (pH 7.3) supplemented with 10% fetal calf serum (FCS), 2.2 g/liter $NaHCO_3$, 10 mM HEPES, 2 mM glutamine, 2.5 mg/liter Amphotericin B, 105 U/liter penicillin-streptomycin, 5 mg/liter insulin, 5 mg/liter transferrin, 20 mg/liter sodium selenite, and 30 pM T_3 until they were confluent. The stable clones were maintained in selection medium with the antibiotic G418 (600 mg/ml). For proliferation and hormone secretion experiments, the cells were washed twice with PBS, and then medium was replaced by an experimental medium consisting of the same supplemented DMEM without FCS. Before and after the stimulation period, cell viability was routinely controlled to ensure that this parameter did not change during the experiment. Cell viability was determined microscopically after ethidium bromide/acridine orange staining.

Constructs

The murine gp130 expression vector (pBMG gp130) was generously provided by Dr. Matsumoto Hibi (24). The cDNA full-length (3-kb *Xho*I/*Xho*I fragment) was subcloned into pcDNA3 (Invitrogen) (pcDNA3/gp130 sense). The antisense expression vector was generated by subcloning of 0.9-kb *Xho*I/*Bam*HI fragment into pcDNA3 expression vector in reverse orientation (pcDNA3/gp130 antisense). cDNA insertions were verified by sequencing.

To measure STAT3 activity, the p4xm 67 TATA-tk-LUC was used, provided generously by Dr. J. E. Darnell, Jr. (25). (DEI) 4-alb-LUC construct, used to evaluate NF-IL-6 activity was kindly provided by Dr. P. Johnson and consists of CCAAT/enhancer-binding protein (C/EBP) responsive DEI sites and an albumin minimal promoter coupled to the luciferase (LUC) gene (26). The LUC reporter gene under the control of 2.5 kb of the PRL promoter (PRL-LUC) construct was provided by Dr. A. Gutierrez-Hartmann (27).

GH3 gp130 stable clones

To obtain stable clones with over- or downexpressing gp130 protein, pcDNA3/gp130 sense, pcDNA3/gp130 antisense, or pcDNA3-control vector were used to transfect GH3 cells by Lipofectamine (Invitrogen). After 48 h of incubation, cells were subcultured and maintained in selection medium containing G418. The pcDNA3/gp130 sense, pcDNA3/gp130 antisense, and pcDNA3 stably transfected clones were denominated gp130-sense (gp130-S), gp130-antisense (gp130-AS), and GH3 control, respectively. The levels of gp130 in these clones were tested by Western blot. Southern blot analysis were performed and confirmed the presence of integrated transgenes in all selected clones, which are independent (*i.e.* the different clones carry gp130 cDNA in different regions of their genome). Results from the same representative clone for each construct are shown in all experiments. Similar results were observed with the different individual clones for each construct as indicated for each experiment.

Western blot analysis

Cells were washed once with PBS (pH 7.0), and then cell lysates were prepared in standard cracking buffer and boiled for 3 min. Equal levels of protein (30 μg) were analyzed by 8% SDS-PAGE. Proteins were blotted onto nitrocellulose Western blotting membranes (Sigma) using standard procedures. A specific anti-gp130 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) previously used by others (28) was used. The antibodies were detected with alkaline phosphatase-conjugated antirabbit IgG (Santa Cruz Biotechnology, Inc.) followed by 5-bromo-4-chloro-3-indoly phosphate p-Toluidine and nitroblue tetrazolium chloride detection (Invitrogen).

Northern blot analysis

Total RNA extraction and Northern blot analysis were performed as described previously (29). Reagents were from Sigma, Roche Molecular Biochemicals (Mannheim, Germany), or Pharmacia (Uppsala, Sweden). Briefly, total RNA, isolated by the guanidine isothiocyanate phenol-chloroform extraction method, was denatured with glyoxal, electrophoresed on a 1.2% agarose gel, and transferred overnight to a nylon membrane. Filters were baked for 2 h at 80 C and stained with methylene blue. They were prehybridized for 1 h at 60 C (50% formamide, 5× sodium chloride/sodium phosphate/EDTA, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, 100 μg/ml denatured salmon sperm DNA), and then the probe was added and hybridized for 12 h. Blots were washed at increasing salt and temperature stringency with a final wash of 30 min at 60 C in 0.1× saline sodium citrate buffer containing 0.1% sodium dodecyl sulfate. After washing, blots were exposed to XAR5 film (Kodak, Rochester, NY) at −70 C with intensifying screens for 6 d. A 0.5-kb SOCS-3 cDNA fragment (30) and a 1-kb *Pst*I fragment of β-actin cDNA (31) were labeled with a random-priming kit using α-^{32}P-dCTP (specific activity, 2–4 × 10^8 cpm/μg). The blots were reprobed after stripping the first probe with 5 mM Tris/HCl (pH 8.0), 2 mM EDTA, and 0.1× Denhardt's solution at 65 C for 2 h. After the previous signal was removed and confirmed by reexposure of the filter, the blots were prehybridized and hybridized following the methods described above. The loading control with the fragment of β-actin cDNA as probe was performed in each blot. The mRNA levels were quantified by using phosphor imager analysis (Fuji Photo Film Co. Ltd., Aichi, Japan).

Cell proliferation

Cell proliferation was measured with the WST-1 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions, and the reaction product was measured in an ELISA plate reader at 450 nm (8, 32). Results were validated by total cell count. Acridine orange-ethidium bromide staining was used to rule out toxic effects.

Hormone determination

Hormones were measured by RIA as previously described (8, 19). For recombinant GH and PRL, reagents were kindly provided by Dr. A. F. Parlow from the National Hormone and Pituitary Program (Torrance, CA).

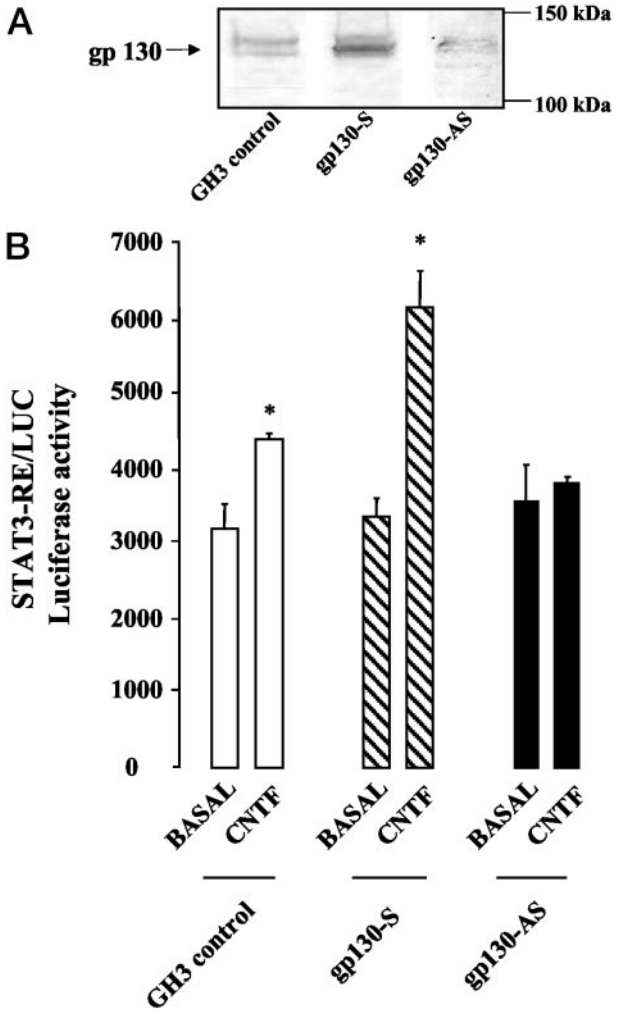

FIG. 1. Characterization of gp130-S and gp130-AS stable clones. A, Western blot analysis of GH3 lactosomatotrophic stable clones showing overexpressed or downexpressed gp130 protein levels. The bands corresponding to gp130 protein were detected by an anti-gp130 polyclonal antibody and are indicated by an *arrow*. GH3 control, GH3 cells transfected with pcDNA3/control vector; gp130-S, GH3 cells transfected with pcDNA3/gp130 sense vector; gp130-AS, GH3 cells transfected with pcDNA3/gp130 antisense vector. Molecular weight marker is indicated on the right. Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown. B, STAT-3-mediated transcriptional activation in response to CNTF in gp130-S and gp130-AS stable clones. GH3 stable lines were transiently transfected with plasmid DNA mixture containing 1.5 μg luciferase reporter construct [(4x) IRF-1] with four copies of the STAT-3-binding sequence from the interferon regulatory factor 1 (IRF-1) and 0.5 μg pRSV-Lac Z. At 20 h after transfection, cells were treated with CNTF (2 nM) *(bars)* for 6 h. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Results are expressed as mean ± SEM (n = 4) of one representative of three independent experiments with similar results. By ANOVA with Scheffé's test: *, $P < 0.05$ (*vs.* corresponding basal). Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown.

Transfection of GH3 cells and reporter assays

Cell transfection was performed with Lipofectamine using standard procedures, as previously described (32). After plating the cells in 6-well plates, the cells were transfected in DMEM without FCS for 6 h using

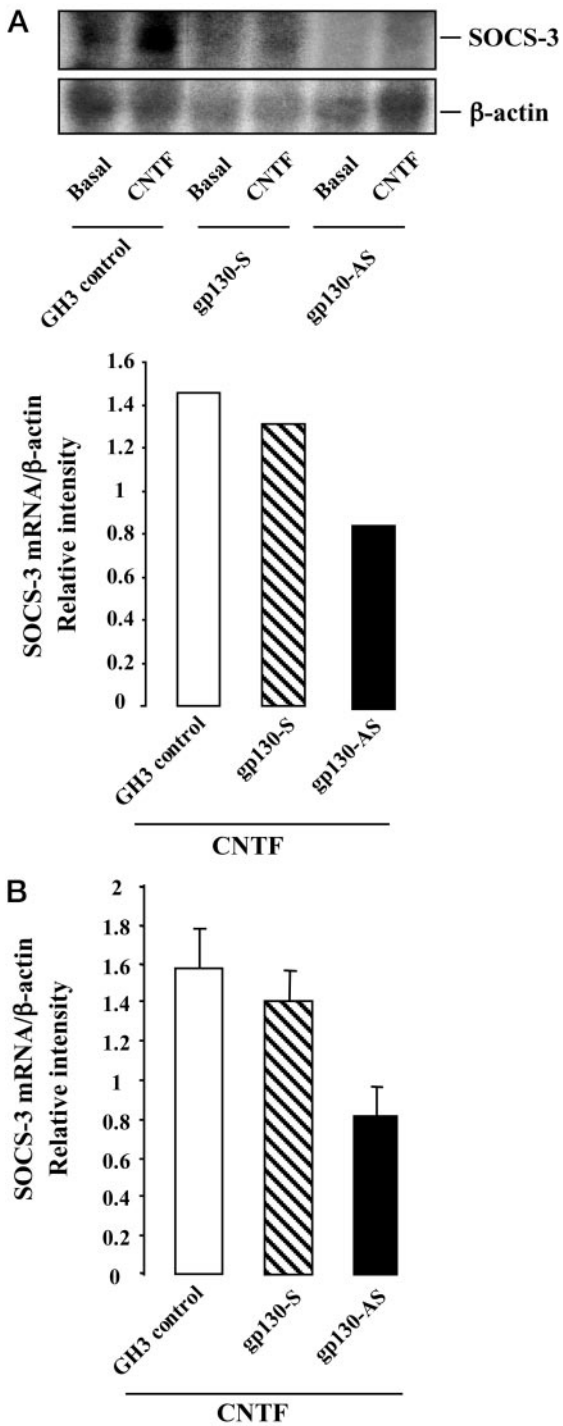

FIG. 2. Effect of CNTF on SOCS-3 mRNA expression in GH3 control, gp130-S, and gp130-AS clones. Northern blot analysis is shown using 30 μg RNA per lane as stated in *Materials and Methods.* A, Bands corresponding to SOCS-3 mRNA (3.2 kb) or β-actin from one representative experiment are shown. The cell lines were seeded at 6×10^5 cells/well and treated with CNTF (2 nM). Densitometric units of the bands relative to constitutive β-actin expression are shown. B, Mean densitometric analysis of SOCS-3 mRNA content from three independent experiments including the one shown in A. Data are expressed as a ratio ± SEM of autoradiographic signals of SOCS-3 mRNA relative to β-actin mRNA used as loading control. Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown.

5 μl lipofectamine (Invitrogen) per well with 1.5 μg for each plasmid, in conjunction with a control Rous sarcoma virus (RSV)-βgal construct (0.5 μg). Cells were then washed and left 20 h in DMEM supplemented with 10% FCS. Then cells were washed and stimulated for 6 h with CNTF (2 nM) in DMEM supplemented with 2% FCS. After treatment, LUC activity in cell lysates was measured as previously described (32) using the Luciferase measure kit (Promega Corp., Madison, WI) with a Junior luminometer (Berlthod, Bad Wildbad, Germany). The β-gal activity was measured as previously described (32) as control for transfection efficiency. Results were standardized for β-gal activity.

In vivo experiments in nude mice

GH3 cells were collected, washed twice with PBS, resuspended in DMEM, and injected (1×10^6 cells) into the flanks of adult (20 g) female N:NIH nude mice. The mice were divided into three groups: injected with GH3 control cells, injected with gp130-S cells, and injected with gp130-AS cells. Animals were examined for tumor formation every 2 d, and tumor growth was determined as described (32). The experiments were finished at 26 d after cell injection because of GH3 control mice morbidity.

All experimental protocols were approved by the Ethical Committee on Animal Care and Use, University of Buenos Aires, Argentina.

Immunohistochemistry for CD31 and determination of vascular density

For immunohistochemistry, 8-μm sections of shock-frozen GH3 tumor tissue were thaw mounted onto SuperFrost Plus slides (Menzel-Glaser, Hamburg, Germany), fixed in 4% paraformaldehyde in PBS, and stored in 96% ethanol at 4 C until use.

An antimouse CD31 (PECAM-1) antibody (Santa Cruz Biotechnology, Inc.) was used to detect intratumoral microvessels. Slides were first incubated in horse serum diluted 1:10 in Tris-based buffer (pH 7.6). Subsequently the slides were incubated overnight at 4 C with CD31 primary antibody diluted 1:500. After three washes in Tris-based buffer, the biotinylated horse antigoat secondary antibody (dilution: 1:300) (Vector Laboratories Inc., Burlingame, CA) was added for 30 min at room temperature. The slides were then washed again and were incubated for 30 min with the avidin-biotin-peroxidase complex (Vector Laboratories Inc.). Color development was performed using 1 mg/ml diaminobenzidine (Sigma) with 0.01% hydrogen peroxide applied for 45 sec. After washing in deionized water, the sections were counterstained with toluidine blue, fixed in Roti-Histol (Carl Roth, Karlsruhe, Germany) and coverslipped using Roti-Histokitt (Carl Roth). Negative controls were performed by omitting the primary antibody. No staining for CD31 has been detected in negative control cells.

For determination of vascular density, an investigator who was not informed about the type of tumor counted vessels inside an area delimited by an eyepiece graticule 12.5 × 12.5 mm divided in 10 × 10 squares (Zeiss, Munich, Germany) at a magnification ×200 (×20 objective and ×10 ocular). Vessel density was determined in six different tumors of each group (gp130-AS; gp130-S; GH3 control-derived tumors), and in each tumor both single cells and cell clusters positive for CD31 were counted in three different areas. Results of vessel counting are expressed as mean ± SEM of 18 areas counted.

Statistics

Statistics were performed by ANOVA in combination with the Scheffé's test. Data are shown as mean ± SEM.

Results

Generation and characterization of gp130 stable cell lines

To generate lactosomatotrophic GH3 stable clones that over- or downexpress the gp130 glycoprotein, GH3 cells were transfected with vectors containing gp130 cDNA in sense or antisense orientations. As shown in Fig. 1A, gp130 glycoproteins were expressed at higher levels in gp130-S clones, whereas they were downexpressed in gp130-AS clones, compared with GH3 cells stably transfected with control vector.

To test the signaling pathway downstream of gp130 in the stably transfected GH3 clones, they were transiently transfected with the STAT3-LUC construct. As shown in Fig. 1B, CNTF (2 nM) induced STAT3-transcriptional activity with respect to the basal condition in the GH3 control cells, which was slightly enhanced in the gp130-S clones. However, in the case of gp130-AS clones, LUC activity was efficiently blocked (Fig. 1B). Similar results were obtained with a C/EBP-LUC reporter construct (data not shown).

In addition, SOCS-3 mRNA was also stimulated in GH3 control and gp130-S clones in response to CNTF, whereas it was blunted in gp130-AS clones (Fig. 2).

Cell proliferation and hormone secretion inhibition in gp130-AS clones

No differences in basal proliferation were observed among different clones (Fig. 3). CNTF significantly stimulated the growth of GH3 control cells, at the same doses previously reported (8), and a similar response was found in the gp130-S clones. In contrast, as shown in Fig. 3, the capacity of the gp130-AS clones to respond to the gp130 specific stimuli was abolished, whereas they were able to respond to a gp130-independent stimulus, TRH. Similar results were obtained in

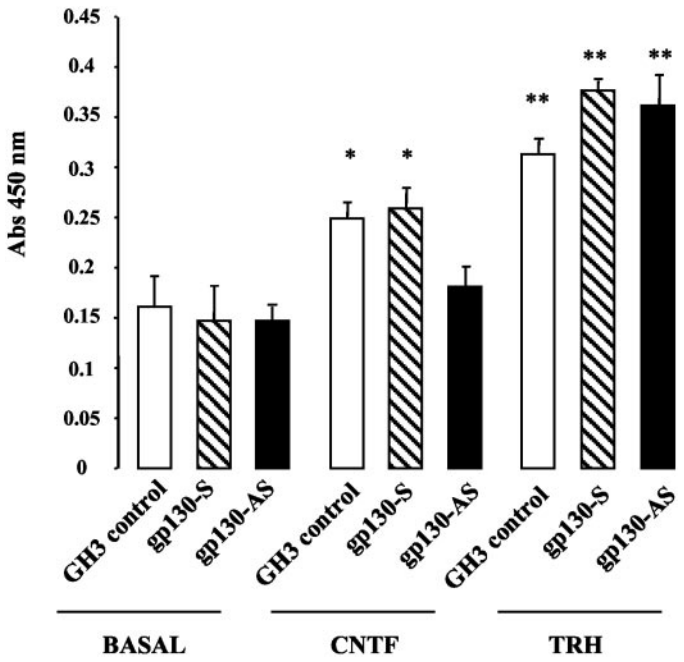

FIG. 3. Proliferation of GH3 control, gp130-S, gp130-AS clones in response to CNTF and TRH. GH3 gp130 cell lines were seeded at 9000 cells/well in multiwell plates with 10% FCS. After attachment, cells were washed twice with PBS and incubated in FCS-free medium for 24 h. Cells were treated either with CNTF (2 nM) or TRH (100 nM) for 72 h. Proliferation was measured by the WST-1 colorimetric method as detailed in *Materials and Methods.* Values represent the mean ± SEM of quadruplicate determinations of a single representative experiment (total of five with similar results). By ANOVA with Scheffé's test: *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding basal). Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown.

the hormone secretion experiments: Both CNTF and TRH significantly stimulated GH secretion from GH3 control and gp130-S cells, whereas the gp130-AS clones had a normal response only to TRH (Fig. 4).

However, gp130-AS clones showed a very low level of basal PRL secretion. Interestingly, the basal activity of the PRL promoter was blunted in the gp130-AS clones, showing they have a constitutive lower basal PRL-transcriptional activity (Fig. 5). No difference between GH3 control and gp130-S cells was observed in terms of hormone secretion (GH and PRL) and cell proliferation stimulated by either gp130 cytokine or TRH (Figs. 3–5).

In vivo tumor formation capacity is inhibited in gp130-AS clones

Based on the findings that GH3 gp130-AS cells had an impaired response to gp130 cytokine stimulation, we hypothesized that the capacity of gp130-AS cells to develop tumors would be inhibited. Therefore, we evaluated the tumor formation by injecting GH3 stables clones into nude

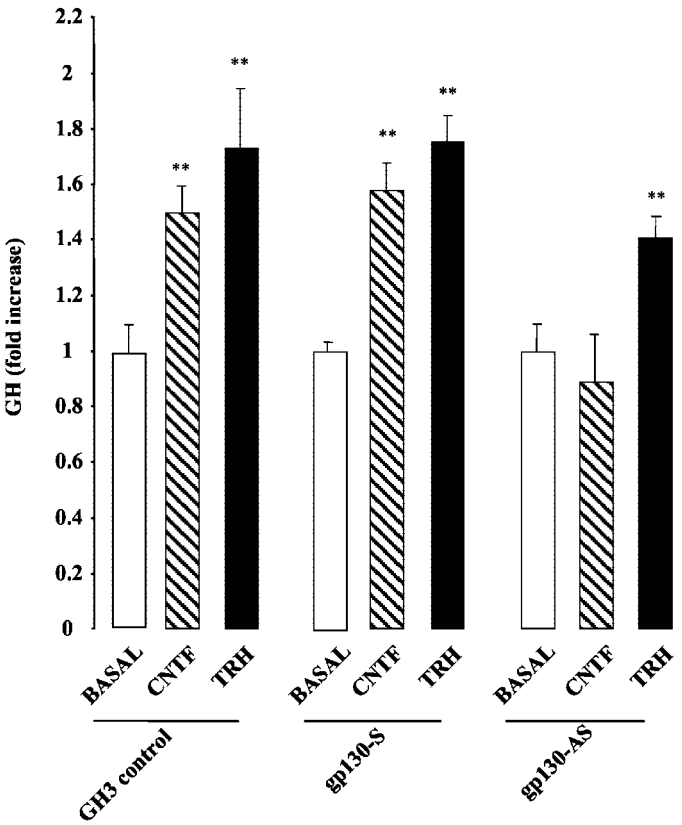

FIG. 4. Effect of recombinant rat CNTF and TRH on GH secretion in GH3 control, gp130-S, and gp130-AS clones. Clones were treated with CNTF (2 nM) or TRH (100 nM) as indicated. GH3 cell lines were seeded at 2×10^5 cells in 24-well plates. After FCS depletion for 24 h, treatments were added with fresh FCS-depleted medium. After 24 h, the supernatants were collected, and GH was measured by RIA. Values represent the mean ± SEM of quadruplicate determinations of a single representative experiment (total of three with similar results). By ANOVA with Scheffé's test: *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding basal). Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown.

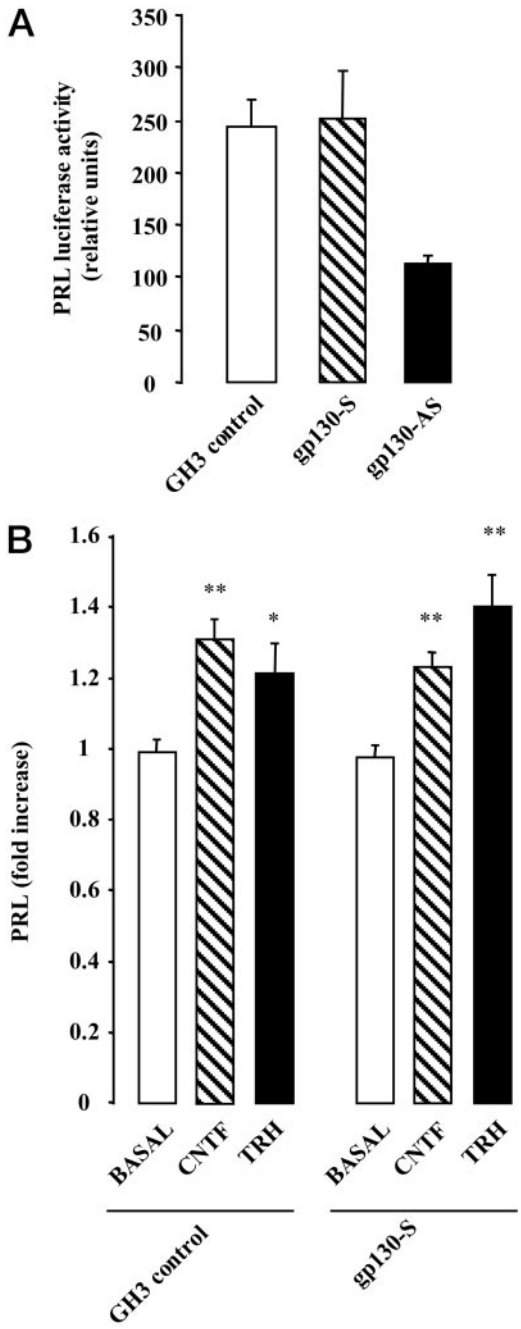

FIG. 5. PRL promoter activity and PRL secretion in GH3 control, gp130-S, and gp130-AS clones. A, GH3 stable lines were transiently transfected with plasmid DNA mixture containing 1.5 μg luciferase reporter construct of PRL-LUC and 0.5 μg pRSV-Lac Z. The values corresponding to luciferase were normalized with respect to β-galactosidase activity, and results are expressed as mean ± SEM (n = 3) of one representative experiment of three independent experiments with similar results. B, Clones were treated with CNTF (2 nM) or TRH (100 nM) as indicated. GH3 cell lines were seeded at 2×10^5 cells in 24-well plates. After FCS depletion for 24 h, treatments were added with fresh FCS-depleted medium. After 24 h, the supernatants were collected and PRL was measured by RIA. Results are expressed as mean ± SEM (n = 4) of one representative of three independent experiments. By ANOVA with Scheffé's test: *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding basal). Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown.

mice. Within 14 d after injection, visible tumors were developed in mice injected either with GH3 control or GH3 gp130-S cells. These tumors grew very rapidly and reached considerable sizes at 26 d after injection (Fig. 6). On the contrary, in mice injected with gp130-AS cells, tumor formation was delayed until 23 d after injection, and they developed tumors of very small sizes. Thus, the growth rate and the tumor size were greatly reduced in comparison with both GH3 control and GH3 gp130-S groups (Fig. 6).

The counting of cells and cell clusters positive for the endothelial cell marker CD31 showed that the vessel density was significantly reduced in gp130-AS-derived tumors. Whereas the vessel density was similar in GH3 control-derived tumors (37.1 ± 5.4 vessels per area) and gp130-S derived-tumors (43.1 ± 6.4 vessels per area), only 12.9 ± 4.5 vessels per area could be detected in gp130-AS derived-tumors (Fig. 7).

Discussion

Pathogenesis of pituitary tumors has been extensively studied to identify those activating oncogene mutations and/or inactivating tumor suppressor genes involved in tumorigenesis (33, 34). For example, G protein (Gsα) mutations have been shown to occur only in a subset of sporadic GH-secreting pituitary adenomas, which represent a small percentage of all pituitary tumors (33, 34), suggesting that there may be other events responsible for pituitary tumorigenesis. A novel pituitary tumor-transforming gene involved in the generation of pituitary adenomas has been cloned from rat GH4 pituitary tumors (35). However, despite these discoveries, it is still an open question how pituitary tumors are formed during pathogenesis.

Several studies have postulated the possibility that gp130 cytokines, through the gp130 glycoprotein-mediated signal transduction cascades, could play an important role in the tumorigenic process in different tissues (36–39). In agreement with this, IL-6 and IL-11 have been reported to stimulate growth of pituitary cells (8, 19–21) and release of angiogenic factors, such as VEGF by FS cells and TtT/GF cells (8, 40). Thus, the importance of the gp130 cytokines in function and growth of pituitary cells could indicate that some genetic events that occur during pituitary pathogenesis

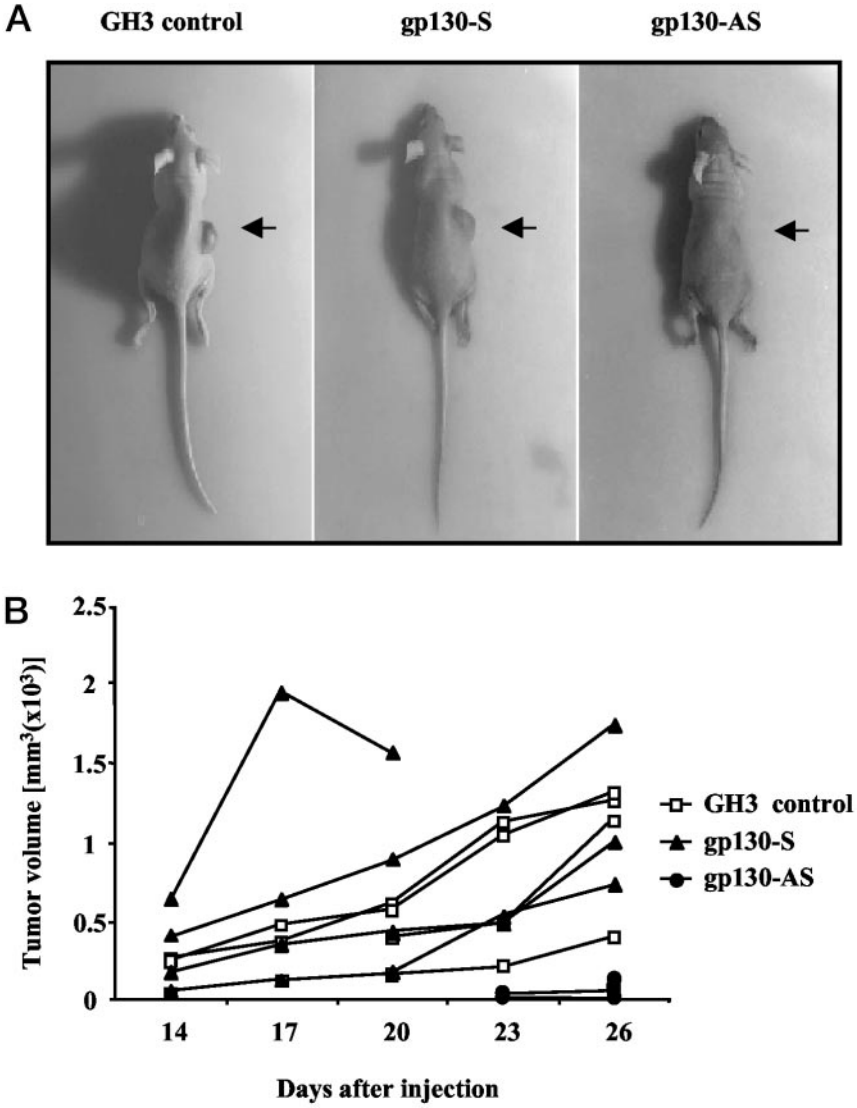

FIG. 6. GH3 cell tumor development is abolished in gp130-AS clones. A, Cells (1×10^6) were injected sc into athymic nude mice (n = 4, each group with the same clone) as described in *Materials and Methods*. Photographs were taken at d 26 after injection. B, The development of tumors in GH3 control and gp130-S-injected animals started to be apparent at d 14 after injection, whereas group gp130-AS showed delayed tumor formation (visible at d 23). In some cases, *symbols* corresponding to individual animals overlap. There were no significant differences between d 26 and d 36 in the animals injected with gp130-AS cells (data not shown). Results from one representative clone (same in all figures) from several gp130-S (n = 2) and gp130-AS (n = 3) stable clones with similar results are shown. The clones preserved the expression of gp130-S or gp130-AS after *in vivo* injection.

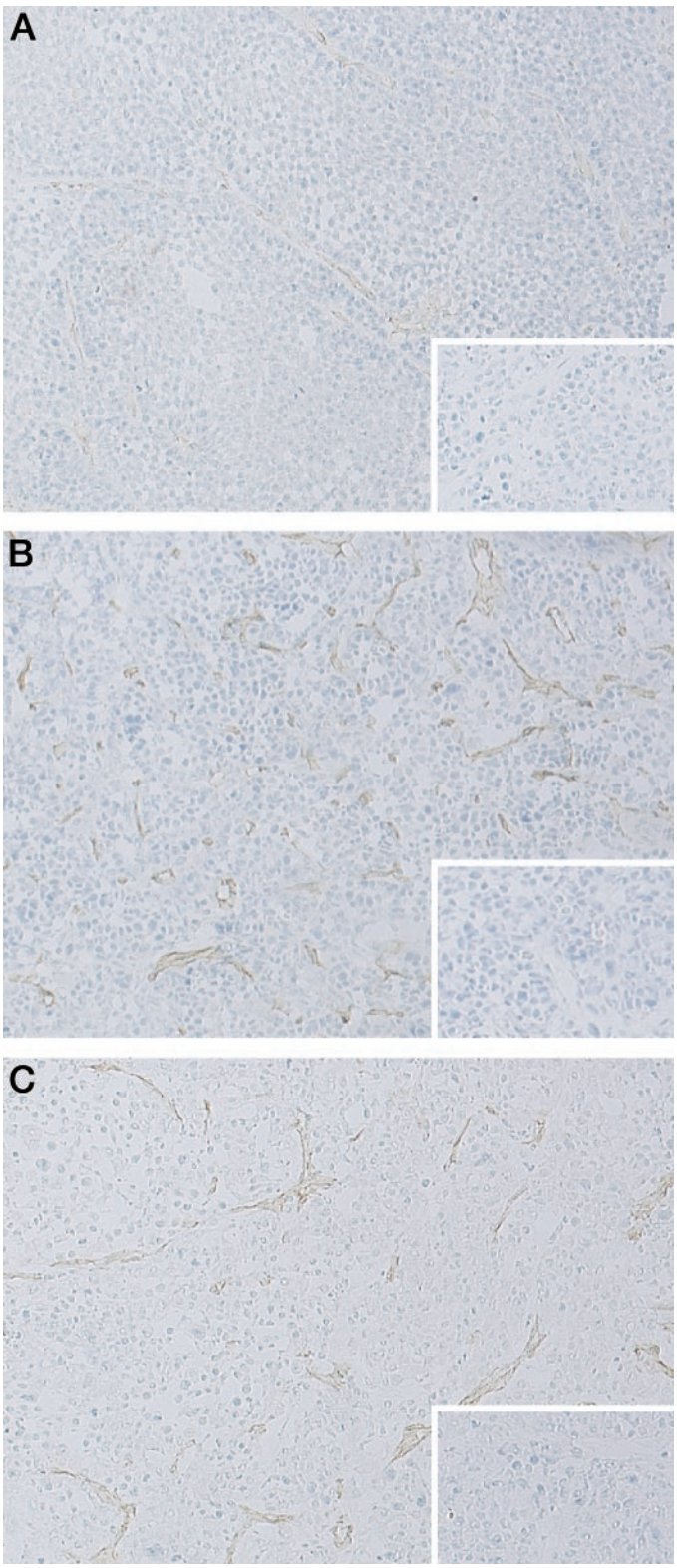

FIG. 7. Density of microvessels in gp130-AS-derived tumors (A) in gp130-S-derived-tumors (B) and GH3 control-derived-tumors (C). Immunostaining for the endothelial cell marker CD31 *(brown)* is shown. The vessel density was significantly reduced ($P < 0.001$) by ANOVA with Scheffé's test in gp130-AS-derived-tumors (with respect to the gp130-S-derived-tumors and GH3 control-derived-tumors). Corresponding negative controls are inserted at the *right corner* of each picture.

would be mediated by signaling response originated from gp130 cytokines. Therefore, the gp130 cytokines should be considered as feasible candidates to generate a favorable ambient for cell transformation events in the pituitary gland. In fact, in the present study, we demonstrated that reducing gp130 level inhibits tumor development of GH3 cells *in vivo*.

Koyama *et al.* (22) have shown that the MtT/S cell line needs the presence of TtT/GF to induce formation of tumor in nude mice. Although it is still uncertain how TtT/GF cells regulate MtT/S's cell behavior, it is likely that the microenvironment determined by cellular interactions and extracellular matrix modifications would contribute to support their tumor development. In contrast, GH3 cells have innate capacity to develop tumors in nude mice (41–43). They respond to angiogenic and growth factors and do not need the presence of other pituitary cells (*i.e.* TtT/GF cells). Hence, GH3 cells represent a good model to study the influence of gp130 protein expression levels during tumor formation. Our results show that GH3 gp130-AS cells have lower gp130 signal cascade activation, showing reduced STAT-3 and C/EBP-activity and also SOCS-3 mRNA expression levels. It is important to notice that GH3 gp130-AS cells are still able to respond to gp130-independent stimuli, such as TRH, which indicates that the lower amount of gp130 protein is indeed specific and critical for gp130 cytokine actions. These clones have reduced proliferative activity. In accordance with our results, altered forms of gp130 that fail to induce activation of JAKs fail to transduce an IL-6 proliferative signal (44); in mouse B cells and an erythroleukemia-derived cell line expressing human gp130 glycoprotein, the presence of gp130 monoclonal antibodies completely abolished the biological response such as the cell growth induced by gp130 cytokines (45).

On the contrary, the overexpression of gp130 did not significantly modify the cellular behavior, which might indicate that pituitary gp130 endogenous levels fulfill for a normal functional cellular response. Similarly, in transgenic mice that overexpress gp130, the activation-signaling pathway downstream of gp130 in the heart is not enhanced (46), suggesting that gp130 overexpression does not always induce changes in the cell biology, which is in accordance with our results.

In the GH3 gp130-S-derived-tumors, vessel density was the highest, observing 2- to 3-fold more vessels in GH3 gp130-S-derived tumors, compared with GH3 gp130-AS-derived tumors. It is therefore clear that in GH3 gp130-AS-derived-tumors, neovascularization is impaired. However, we need to address further studies to determine whether this is a direct effect (suppression of gp130-mediated VEGF and/or basic fibroblast growth factor production because of gp130 blockade) or an indirect effect because of lower tumor cell growth associated with lack of tumor expansion and hypoxia-induced neoangiogenesis.

We show for the first time that the reduced level of gp130 protein in GH3 cells (gp130-AS cells) blocked the cell growth and hormone secretion stimulated by CNTF and have impaired tumor formation capacity in nude mice. These data contribute to support gp130 cytokines as candidates that would favor the development and/or growth of pituitary tumors.

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