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Human Somatostatin Receptor Subtypes in Acromegaly: Distinct Patterns of Messenger Ribonucleic Acid Expression and Hormone Suppression Identify Different Tumoral Phenotypes*

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ABSTRACT

Recently, studies using somatostatin (SRIF) analogs preferential for either the SRIF receptor 2 (SSTR2) or the SSTR5 subtype demonstrated a variable suppression of GH and PRL release from GH-secreting human adenomas. These data suggested the concept of SSTR subtype specificity in such tumors. In the present study the quantitative expression of messenger ribonucleic acid (mRNA) for the 5 SSTR subtypes and the inhibitory effects of SRIF14; SRIF28; octreotide; the SSTR2-preferential analog, BIM-23197; and the SSTR5-preferential analog, BIM-23268, on GH and PRL secretion were analyzed in cells cultured from 15 acromegalic tumors. RT-PCR analysis revealed a consistent pattern of SSTR2 and SSTR5 mRNA expression. SSTR5 mRNA was expressed at a higher level (1052 ± 405 pg/pg glyceraldehyde-3-phosphate dehydrogenase) than SSTR2 mRNA (100 ± 30 pg/pg glyceraldehyde-3-phosphate dehydrogenase). However, only SSTR2 mRNA expression correlated with the degree of GH inhibition induced by SRIF14, SRIF28, and BIM-23197. The SSTR5-preferential compound inhibited GH release in only 7 of 15 cases.

In cells cultured from the 10 mixed adenomas that secreted both GH and PRL, RT-PCR analysis revealed a consistent coexpression of SSTR5, SSTR2, and SSTR1 mRNA. In all cases SRIF14, SRIF28, and the SSTR5-preferential analog, BIM-23268, significantly suppressed PRL secretion, with a mean maximal inhibition of 48 ± 4%. In contrast, the SSTR2-preferential analogs, BIM-23197 and octreotide, were effective in suppressing PRL in only 6 of 10 cases. In cells cultured from adenomas taken from patients partially responsive to the SRIF analog, octreotide, partial additivity in suppressing both GH and PRL secretion was observed when the SSTR2- and SSTR5-preferring analogs, BIM-23197 and BIM-23268, were tested in combination. Our data show a highly variable ratio of the SSTR2 and SSTR5 transcripts, according to tumors. The SSTR2-preferring compound consistently inhibits GH release, whereas the SSTR5-preferring compound is the main inhibitor of PRL secretion. When both drugs are combined, the partial additivity observed in mixed GH- plus PRL-secreting adenomas may be of interest in the therapeutic approach of such tumors. (*J Clin Endocrinol Metab* **85:** 781–792, 2000)

SOMATOSTATIN (SRIF) exerts its biological effects via five distinct high affinity membrane receptor (SSTR) subtypes that belong to the family of G protein-coupled receptors (1). Human GH-secreting adenomas appear to variably express the different SSTR subtypes (2–4). Such quantitative variations have been cited to explain the variable efficacy of SRIF agonists, such as octreotide and lanreotide, in the medical treatment of acromegaly (5–7). Recent studies using SRIF analogs that are preferential for the human SSTR2 and SSTR5 receptor subtypes, such as BIM-23197 and BIM-23268, respectively, suggest the involvement of both SSTR2 and SSTR5 in regulating GH secretion from somatotroph adenomas, whereas SSTR5 mainly suppresses PRL secretion from prolactinomas (8, 9). Using cells cultured from either GH-secreting or mixed GH- and PRL-secreting (GH+PRL) adenomas taken from 15 acromegalic patients, the present study was undertaken to address the following questions. 1) What is the quantitative pattern of expression of the five SSTR messenger ribonucleic acids (mRNAs)? 2) Does it differ between pure and mixed GH- and PRL-secreting adenomas? 3) Using SSTR2- and SSTR5-preferential analogs, to what degree do these SSTR subtypes suppress GH and PRL secretion? 4) In tumors from acromegalic patients that are only partially responsive to octreotide or lanreotide therapy, can we observe any additive effect of the combination of both preferential agonists in the suppression of GH and PRL release?

Our data reveal a similar pattern of SSTR2 and SSTR5 mRNA expression in both pure and mixed adenomas. Despite its lower expression, SSTR2 mRNA expression correlated significantly with the GH-suppressive effects of SRIF14, SRIF28, and BIM-23197. SSTR1 transcripts were only expressed in tumors secreting PRL. The SSTR5-preferential compound, BIM-23268, is the dominant inhibitor of PRL in the mixed adenomas. Therefore, due to the different inhib-

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itory actions regulated by the two receptor subtypes, a combination of both preferential compounds may allow a better control of hormones hypersecretion in these mixed adenomas.

Subjects and Methods

Patients

The present study was approved by the ethics committee of the University of Aix-Marseilles (Marseilles, France) and was undertaken after obtaining informed consent from each patient. Fifteen acromegalic patients (eight women and seven men), aged 18–66 yr, presenting with a macroadenoma were studied. Their endocrine status and the neuroradiological characterization of the tumors were documented before any treatment. Basal GH levels were expressed as the mean of consecutive measurements obtained hourly between 0800–1300 h. SRIF agonist sensitivity was assessed by an acute test using a single sc 200-μg injection of octreotide (Sandostatin, Novartis, Basel, Switzerland). Blood samples were withdrawn hourly before and for 6 h after octreotide treatment to measure GH variations. Sensitivity to octreotide was expressed as the percent decrease in GH from basal to the mean GH value 2–6 h after octreotide injection. The basal insulin-like growth factor I (IGF-I) value was evaluated under fasting conditions between 0800–0900 h. Magnetic resonance imaging revealed adenomas with a maximal 11- to 48-mm diameter either enclosed in the pituitary fossa (n = 8) or with an extension toward the adjacent structures, mainly the cavernous sinus (n = 7). All patients underwent transsphenoidal surgery. The clinical endocrine and tumoral status of each patient is summarized in Tables 1 and 2, which are divided according to classification as pure GH-secreting adenomas or mixed GH+PRL adenomas. The definition of the later subgroup was not based upon basal plasma PRL values, but was established according to the 24-h release of both GH and PRL on day 3 of cell culture, as shown in Table 2.

Hormone assays

GH and PRL were measured using commercial immunoradiometric kits (Immunotech, Marseilles, France). Normal GH values ranged from 0.05–2.4 μg/L; normal PRL values ranged from 1–24 μg/L in women and from 1–17 μg/L in men. After an ethanol-acid extraction, the plasma IGF-I assay was performed using the IGF-I RIA kit from Nichols Institute Diagnostics (San Juan Capistrano, CA). The normal ranges, according to sex and age, were established by our laboratory.

Detection of SSTR subtypes

Total RNA was extracted from 30–60 mg tissue from each tumor using the SV total RNA isolation system (Promega Corp., Lyon, France). The RNA samples were subsequently treated with 30 U ribonuclease-free deoxyribonuclease I (Roche Molecular Biochemicals, Mannheim, Germany) to prevent any contamination by genomic DNA. Total RNA was reverse transcribed into complementary DNA (cDNA) using 1 μg hexamers (Pharmacia Biotech, Orsay, France) and Moloney murine leukemia virus reverse transcriptase, as described by the manufacturer.

The 5′-exonuclease (Taq Man) assay, which produces a direct proportional readout for the progression of PCR reactions, was used (10). Amplification of cDNA derived from 50–150 ng total RNA was performed in a 50-μL reaction volume with a buffer consisting of 10 mmol/L Tris-HCl (pH 8.3; 25 C); 50 mmol/L KCl; 10 mmol/L ethylenediamine tetraacetate; 5 mmol/L $MgCl_2$ in the presence of 200 μmol/L deoxy (d)-ATP, dCTP, and dGTP; 400 μmol/L dUTP; 1 μmol/L of each primer; 200 nmol/L probe; 1 U Amp Erase UNG (Perkin-Elmer Corp., Paris, France); and 1.25 U AmpliTaq Gold polymerase (Perkin-Elmer Corp.). The probe comprised 20–30 nucleotides with 5′-end substitution with a fluorophore and a quencher substitution at the 3′-end. The synthetic SSTR cDNA primers used in the PCR reaction were 19- or 20-mers as follows: SSTR1: sense, 1411–1433; antisense, 1511–1492; probe, 1442–1463; SSTR2: sense, 10–29; antisense, 109–91; probe, 58–32; SSTR3, sense, 1188–1206; antisense, 1254–1236; probe, 1209–1234; SSTR4: sense, 1282–1301; antisense, 1362–1343; probe, 1331–1301; and SSTR5: sense, 1103–1119; antisense, 1156–1139; probe, 1137–1121. The annealing-extension temperatures were: for SSTR1, 66 C; for SSTR2, 56 C; for SSTR3, 70 C; for SSTR4, 66 C; and for SSTR5, 70 C. Forty cycles of two-step PCR reaction-annealing extension at specified temperatures for 30 s and denaturation at 95 C for 20 s,were performed on a ABI Prism 7700 sequence detection apparatus (Perkin-Elmer Corp.). For quantitation of data, SSTR mRNA levels were, in the same reaction, normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The control GAPDH primers was as follows: sense, 222–240; antisense, 322–303; and probe, 277–301. For each measurement, three independent RT-PCR analyses were performed. To produce standard curves for each SSTR mRNA and GAPDH mRNA, RNAs were produced by *in vitro* transcription from linearized templates corresponding to SSTRs and GAPDH cDNA constructs using T7 or T3 polymerases as previously described (11). The synthesized RNAs were reverse transcribed to cDNA for each parameter as described above. Using the fluorogenic probes for SSTR receptors and GAPDH with the experimental conditions defined above, we obtained a linear relationship between the RNA concentration (previously transcribed into cDNA) and the fluorescent signal (ΔRQ) for SSTR and GAPDH RNAs in 1–250 pg DNA target. For each unknown sample, we determined the ΔRQ values for both genes, and the results were expressed as picograms of SSTR per pg GAPDH.

Cell culture studies

A portion of each tumor obtained at surgery was dissociated by mechanical and enzymatic methods. Depending on the tumor, 3–30 $\times$ 10^6 isolated cells were obtained. Tumoral cells were initially cultured in DMEM supplemented with 10% FCS for 3 days. On day 3, the cells were washed and plated in multiwell culture dishes coated with extracellular matrix from bovine corneal endothelial cells. When they were attached to the matrix on days 4–6, depending on the culture, the medium was removed and replaced with serum-free DMEM supplemented with antibiotics, insulin, transferrin, and selenium as previously described (12). The effects of various doses of the SRIF agonists, SRIF14, SRIF28, octreotide, BIM-23268, and BIM-23197, on inhibition of GH and PRL release were measured over an 8-h period between days 5–7 of culture. Each drug concentration was tested in quadruplicate culture wells (Costar 3524, Brumath, France). SRIF14 and SRIF28 were purchased from Sigma (Saint-Quentin Fallavier, France). Octreotide was supplied by Novartis (Basel, Switzerland). BIM-23268 and BIM-23197, respectively preferen-

TABLE 1. Clinical characteristics of acromegalic patients (GH pure adenoma)

Case no.	Sex	Age (yr)	Tumor size (mm)[a]	*In vivo*				*In vitro*[b]	
				IGF-I (μg/L)	GH (μg/L)		PRL (μg/L)	GH (μg/day)	PRL (μg/day)
					Basal	Under octreotide[c]			
A1	M	48	18	969	26	5 (81)	8	4.4	0
A2	F	30	20	1139	75	4.1 (94)	17	2.13	0
A3	F	39	18	1187	17	8 (53)	17	1.4	0
A4	M	44	18	1000	100	32 (68)	5	1.4	0
A5	F	56	20	1151	31	17 (46)	8	1.1	0

[a] Maximal tumor diameter evaluated by MRI.
[b] Twenty-four-hour GH and PRL secretion in cell culture (day 3) for 1 million adenoma cells.
[c] Mean GH values 2–6 h after octreotide (200 μg, sc). Percent inhibition is indicated in *parentheses*.

TABLE 2. Clinical characteristics of acromegalic patients (mixed GH-PRL adenoma)

Case no.	Sex	Age (yr)	Tumor size (mm)[a]	*In vivo*				*In vitro*[b]	
				IGF-I (μg/L)	GH (μg/L)		PRL (μg/L)	GH (μg/day)	PRL (μg/day)
					Basal	Under octreotide[c]			
A6	M	31	22	813	34	2 (94)	48	3.7	1.7
A7	M	53	11	1171	11	1 (94)	4	13.3	3.2
A8	F	30	35	1300	105	11 (89)	27	3.3	14.3
A9	M	63	20	595	61	6 (90)	12	1.1	0.8
A10	M	45	15	1302	17	2 (91)	4	4.1	0.3
A11	F	44	13	881	90	ND	52	2.4	0.2
A12	F	29	42	549	185	127 (32)	30	2	1.1
A13	F	25	48	800	143	117 (18)	83	3.3	0.3
A14	F	66	12	972	6	4 (33)	5	1.5	1.8
A15	M	53	25	1184	40	24 (40)	10	0.7	0.5

ND, Not determined.
[a] Maximal tumor diameter evaluated by MRI.
[b] Twenty-four-hour GH and PRL secretion in cell culture (day 3) for 1 million adenoma cells.
[c] Mean GH values 2–6 h after octreotide (200 μg, sc). Percent inhibition is indicated in *parentheses*.

tial for SSTR5 and SSTR2 subtypes (13), were provided by Biomeasure, Inc. (Milford, MA). The human SSTR subtype affinities (IC_{50}; nanomoles per L) of each compound are summarized in Table 3. The native SRIFs and SRIF analogs were dissolved in 0.01 mol/L acetic acid containing 0.1% purified serum albumin (Life Technologies, Inc., Cergy Pontoise, France). The drugs were stored at −80 C as 10^{-3} mol/L solutions. Fresh working solutions were prepared from a new aliquot for each experiment.

Triple in situ hybridization histochemistry and quantitative microscopic analysis

In situ hybridization was performed in 2 mixed GH+PRL adenomas, characterized as either sensitive (A6) or resistant (A15) to the SRIF agonists based upon *in vivo* data and cell culture studies. Sections 12 μm thick were cut in a cryostat microtome at −20 C. They were thaw-mounted onto twice gelatin-coated slides, dried on a slide warmer, and kept at −80 C. The sections were hybridized simultaneously with the SSTR2, SSTR5, PRL, and GH probes, as previously described (14). The SSTR2 probe was a 706-bp fragment of the human SSTR2 cDNA (bases 320-1025) cloned into Bluescript and labeled with [^{35}S]UTP (SA, 1200 Ci/mmol; New England Nuclear, Paris, France) using either T3 (antisense probe) or T7 (sense probe) RNA polymerase. The SSTR5 probe was a 936-bp fragment of the human SSTR5 cDNA (bases 81–1016) cloned into Bluescript and labeled with [^{35}S]UTP (SA, 1200 Ci/mmol; New England Nuclear) using either T3 (antisense probe) or T7 (sense probe) RNA polymerase. The PRL probe was a 588-bp fragment of the human PRL cDNA (bases 94–681) cloned into pPRC script and labeled with digoxigenin-UTP (Roche Molecular Biochemicals, Meylan, France) using T3 (antisense probe) or T7 (sense probe) RNA polymerase. The GH probe was a 558-bp fragment of the human GH cDNA (bases 56–611) cloned into pPRC script and labeled with streptavidin-UTP (Roche Molecular Biochemicals) using either T3 (antisense probe) or T7 (sense probe) RNA polymerase. PRL and GH hybridization signals were revealed using fluorescein isothiocyanate- or rhodamine-tyramide, respectively. Slides were subsequently dipped into nuclear emulsion (1:1 water: K5, Ilford, Saint-Priest, France) and exposed for 2 months for SSTR5 mRNAs and for 4 months for SSTR2 mRNAs. Sections triple hybridized with the corresponding sense riboprobes served as controls. Specimens were viewed under a Leitz DMDR microscope (Wetzlar, Germany) equipped with a planachromatic fluorite 40/0.70 objective. Ten systematic random fields were sampled for each tumor (15), captured with a color CCD camera and digitized through a Neotech Image Grabber (Neotech, Hampshire, UK) slotted on a microcomputer. Each sampled field was captured 4 times: once under epifluorescence with the barrier filter centered at 520 nm (fluorescein isothiocyanate), once similarly with the filter barrier at 600 nm (rhodamine), once under Koehler's illumination transmitted darkfield for detecting the radioautography silver grains, and once under phase contrast to assess the total number of cells in the field (including the unlabeled cells). The corresponding 4 digitized images were overlaid with Photoshop software (Adobe System, Inc. Mountain View, CA). Cells were identified individually and computed according to their label characteristics, *i.e.* unlabeled cells; single labeled cells for GH, PRL, SSTR2, or SSTR5 mRNAs; dual labeled cells for the messengers of 1 hormone and SSTR2 or SSTR5 mRNA; and triple labeled cells for both hormones and either SSTR2 or SSTR5 mRNA. The onus of counting was undertaken by a single investigator. Countings were expressed as the ratio of the percentage of the class to the total number of cells computed in the 10 microscopic fields representing each tumor.

Statistics

The results are presented as the mean ± SEM. Statistical significance between two unpaired groups was determined by the Mann-Whitney test. To measure the strength of association between the pairs of variables, without specifying dependencies, Spearman order correlations were run. $P < 0.05$ was considered significant for all tests.

Results

SSTR subtype mRNA expression in acromegaly

RT-PCR quantitative analysis was performed on tumor fragments of the 15 adenomas. The individual patterns of SSTR2 and SSTR5 mRNA expression are presented in Fig. 1. Based on hormone release by cell cultures prepared from these tumors, the present series included 5 pure GH-secreting adenomas and 10 mixed GH+PRL adenomas. This latter group included 5 tumors from hyperprolactinemic patients (plasma PRL, 27–83 μg/L) and 5 tumors from normoprolactinemic patients but, nevertheless, with significant PRL release (0.3–3.2 μg/day·10^6 cells) *in vitro*, as indicated in Table 2. RT-PCR analysis demonstrated the presence of both SSTR2 and SSTR5 mRNA in all tumors. The mean level of SSTR2 mRNA expression was 100 ± 30 pg/pg GAPDH, whereas the mean level of SSTR5 mRNA was 1052 ± 406 pg/pg GAPDH in the series. The individual pattern of expression was highly variable, depending on the tumor. In particular, 4 tumors (2 pure GH and 2 mixed GH+PRL adenomas) expressed SSTR5 mRNA levels over 1000 pg/pg GAPDH. No tumor expressed only SSTR2 or SSTR5 mRNA alone, although the expression of SSTR5 mRNA was largely dominant in 2 tumors (A4 and A13). Although trends were observed, the expression level of SSTR2 and SSTR5 was not

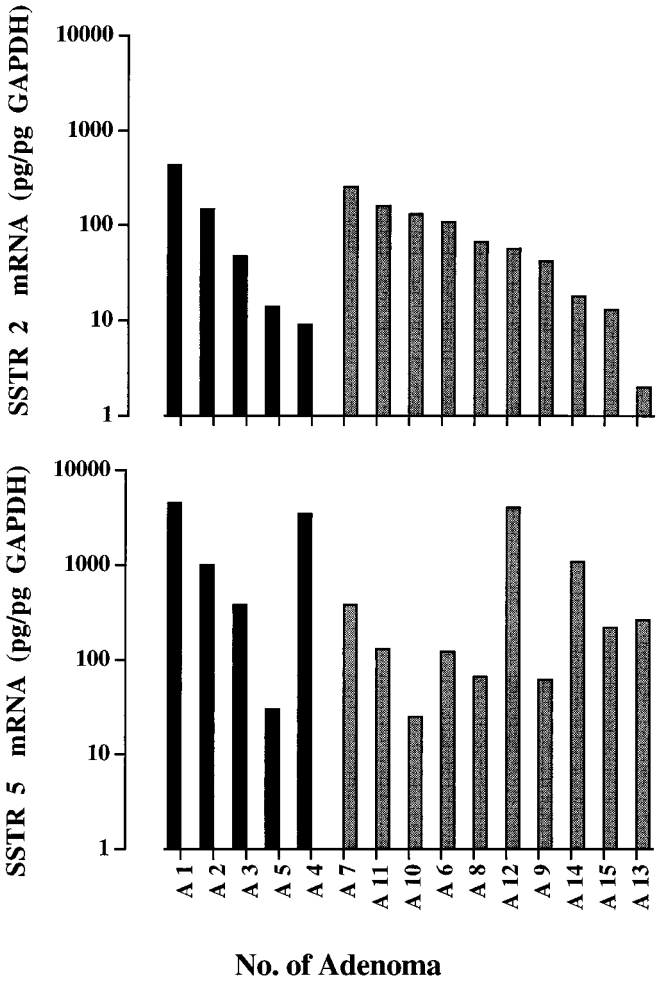

FIG. 1. Quantitative RT-PCR expression of SSTR2 and SSTR5 mRNA in 5 GH pure adenomas (*black bars*) and 10 GH+PRL mixed adenomas (*gray bars*). The tumors were ranked according to the level of SSTR2 mRNA expression in each group. Results are expressed as picograms of SSTR mRNA per pg GAPDH mRNA (mean of 3 runs).

significantly different between the 5 pure GH and the 10 mixed GH+PRL adenomas. In particular, SSTR5 mRNA expression, previously shown to be correlated to the inhibition of PRL release in prolactinomas (9), was greater, although not significantly so, in the pure GH-secreting adenomas (1874 ± 888 pg/pg GAPDH for pure GH-secreting adenomas *vs.* 640 ± 390 pg/pg GAPDH for GH+PRL adenomas). The SSTR1 mRNA, which has been reported to be highly expressed in pure prolactinomas (8, 9), was poorly expressed among the 15 tumors studied (30 ± 18 pg/pg GAPDH). In the majority of tumors, the level of SSTR1 expression ranged from 1–16 pg/pg GAPDH, and it was mainly expressed in the mixed GH+PRL tumors. In only 3 of 10 GH+PRL tumors (A6, 7, and 13) was SSTR1 mRNA expression high, ranging from 43–278 pg/pg GAPDH. SSTR3 mRNA expression was highly variable, being either absent (n = 7) or at a mean expression level of 271 ± 104 pg/pg GAPDH in 8 tumors (A2, 3, 4, 9, 10, 12, 14, and 15). Finally, SSTR4 mRNA expression was not observed in any tumor.

Maximal GH and PRL suppression by SRIF14 and SRIF28

In the cell culture studies, the effects of SRIF14 and SRIF28 on GH and PRL secretion were measured after an 8-h incubation period. Previous experiments (not shown) demonstrated that maximal inhibition of GH, by nanomolar concentrations of these drugs, occurred after 4 h of incubation. Because in some tumors, the basal release of either GH or PRL was low during a 4-h incubation (1–5 ng/medium in control conditions), an 8-h incubation period was adapted to achieve accurate measurements of hormone release from all cultures. As shown in Fig. 2a, the maximal inhibition of GH release by SRIF14, compared with controls (medium alone), ranged from 29–59% among the 5 pure GH-producing adenoma cell cultures and from 25–51% among the 10 mixed GH+PRL adenoma cell cultures. Under the same conditions, SRIF28-induced similar inhibition of GH release in 14 of 15 cases. The mean inhibitory effect of SRIF14 on GH release was 41 ± 3% and was not different from the 39 ± 4% inhibitory effect of SRIF28 at nanomolar concentrations.

In the same experiments, among the 10 mixed adenoma cell cultures the mean maximal inhibitions of PRL release by SRIF14 and SRIF28 were 48 ± 4% and 40 ± 5%, respectively (Fig. 2b). The mean suppressive effect on PRL secretion was positively correlated to the mean maximal GH suppression in the same 10 cultures studies (mean GH suppression by SRIF14 and SRIF28, 47 ± 3% and 38 ± 5%, respectively; $P < 0.02$).

Correlation between SSTR subtype mRNA expression and inhibition of hormone release (GH and PRL)

The degree of GH inhibition by SRIF14 and SRIF28 and the levels of SSTR2 mRNA expression were highly correlated among the 15 tumors analyzed ($P < 0.003$), as illustrated for SRIF14 in Fig. 3a. In the same tumors, despite a much higher expression of SSTR5 transcripts, there was no correlation with the ability of the 2 SRIFs to inhibit GH release, as illustrated for SRIF14 (Fig. 3b). Among the limited number of mixed tumors that cosecreted GH and PRL, no correlation could be demonstrated between the inhibition of PRL release and the expression of either SSTR2 or SSTR5 mRNAs.

Effects of SSTR2- and SSTR5-preferential agonists on GH secretion

The absence of correlation between SSTR5 mRNA expression and the inhibition of GH release by either SRIF14 or SRIF28 prompted us to analyze more precisely the responses of adenoma cells to compounds preferential for the SSTR2 and SSTR5 subtypes. A dose-response inhibition of GH release was examined with 10^{-12}–10^{-8} mol/L concentrations of SRIF14; of the SSTR2-preferring analog, BIM-23197; of the SSTR5-preferring compound, BIM-23268; and of octreotide, the analog used *in vivo* (Tables 1 and 2). The correlation between GH inhibition by octreotide *in vivo* and *in vitro* (with nanomolar concentrations) was highly significant (r = 0.74; $P < 0.008$) in the 15 cases. Among the 15 adenoma cell cultures, 2 patterns of responses to SSTR2- and 5-preferential analogs were observed. In cultures from 7 of 15 tumors (Fig. 4a), the mean dose-response inhibitions of GH release by

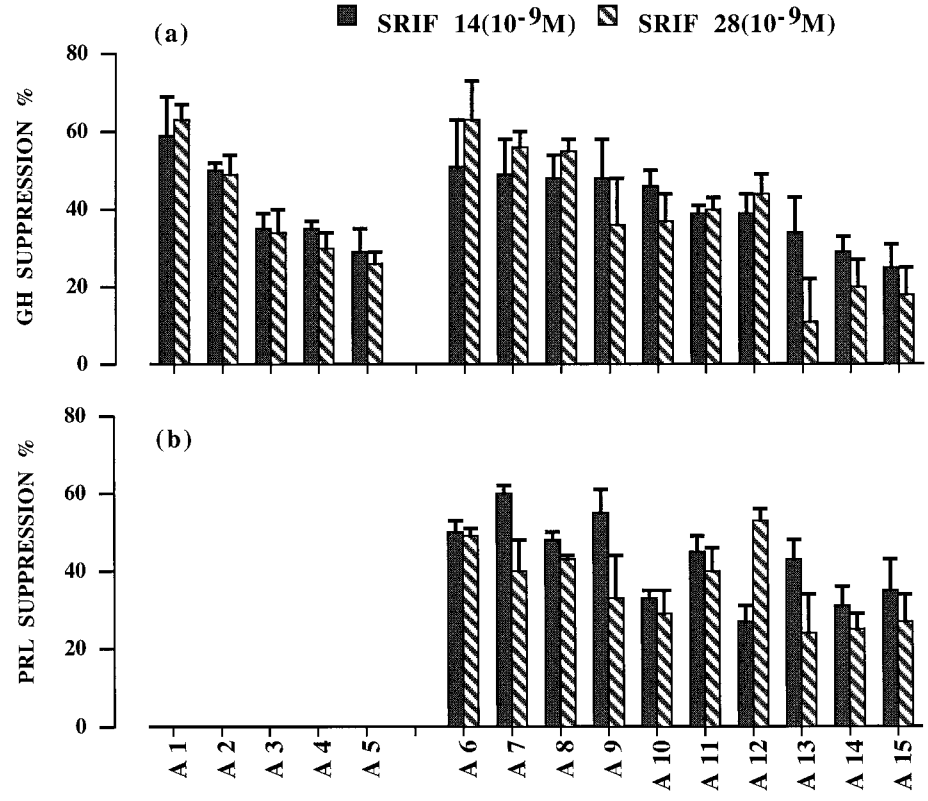

FIG. 2. Maximal GH suppression in 5 pure GH adenomas and maximal GH and PRL suppression in 10 mixed GH+PRL adenomas treated with either SRIF14 (*gray bars*) or SRIF28 (*hatched bars*) at a 1 nmol/L concentration for 8 h. Results are expressed as the percent GH or PRL suppression *vs.* the control value (medium alone). Values are the mean ± SEM of four wells. a, GH suppression; b, PRL suppression.

BIM-23197 and BIM-23268 were identical (EC_{50}, 0.02 ± 0.01 and 0.03 ± 0.01 nmol/L, respectively). Superimposable results were achieved with SRIF14 in these studies. Octreotide realized a similar dose-response inhibition of GH at slightly higher concentrations (EC_{50}, 0.06 ± 0.06 nmol/L) than with BIM-23197 or SRIF14. These 7 cases (A1, 3, 4, 6, 12, 14, and 15) were from both GH only and mixed GH+PRL adenomas. In the others 8 of 15 cultures from the remaining 8 tumors (Fig. 4b), superimposable dose-response inhibition for GH occurred in the presence of BIM-23197 and SRIF14 with a similar EC_{50} (0.02 ± 0.01 nmol/L). In contrast to the previously discussed tumors, the SSTR5 agonist produced a dose-dependant inhibition of GH release at 30-fold higher concentrations (EC_{50}, 0.6 ± 0.2 nmol/L) than required by either SRIF14 or BIM-23197. In the same tumors, octreotide produced a dose-response inhibition for GH with an EC_{50} of 0.1 ± 0.04 nmol/L. The discrepancy between the results obtained with BIM-23197 and BIM-23268 can be explained on the basis of the binding characteristics of BIM-23268 (Table 3), which, at high concentrations, behaves as a weak SSTR2 agonist. Thus, these results reveal 1 class of tumors that are equally responsive to SSTR2- and SSTR5-preferential agonists and a second class of tumors in which the GH-suppressive effect is only mediated through the SSTR2 subtype.

Effects of SSTR2- and SSTR5-preferential agonists on PRL secretion

Dose-response inhibition of PRL secretion by SRIF14, BIM-23197, BIM-23268, and octreotide was also studied in all cultures of the 10 mixed (GH-PRL) tumors. As already shown (Fig. 2) SRIF14 and SRIF28, at nanomolar concentrations, produced a significant inhibition of PRL release from all tumors. In all 10 tumors (Fig. 5), SRIF14 and the SSTR5 preferential analog, BIM-23268, induced a superimposable dose-related suppression of PRL (EC_{50}, 0.09 ± 0.08 and 0.08 ± 0.06 nmol/L, respectively). The dose-related inhibition of PRL by the SSTR2-preferring compound, BIM-23197 (as well as by octreotide), showed 2 markedly distinct patterns. In 6 of 10 tumors (A6, 7, 8, 12, 14, and 15) BIM-23197 and octreotide induced a dose-related suppression of PRL parallel to that of SRIF14, with 5-fold higher concentrations for octreotide (Fig. 5a). In the remaining 4 tumors (Fig. 5b), BIM-23197 and octreotide were unable to significantly suppress PRL release at any concentration. Thus, as observed for GH inhibition, 2 patterns of PRL suppression were revealed by the preferential SSTR2 and 5 compounds. In 6 tumors, PRL suppression was equally achieved through activation of either SSTR5 or SSTR2 subtype, whereas in the remaining 4 tumors PRL inhibition was only mediated by the SSTR5 subtype.

Distinct individual patterns of GH and GH plus PRL suppression by SSTR2- and SSTR5-preferring compounds

The previous data, analyzed as the mean inhibitory effects of BIM-23197 or BIM-23268 on either GH release in the 15 tumors or PRL release from the 10 of 15 mixed adenomas, do not fully reveal the heterogeneous range of responses to the SSTR2 and SSTR5 preferential compounds encountered among individual tumors. From the analysis of the individual dose-response inhibition of GH alone, 2 types of responses were identified in the GH-only secreting adenomas.

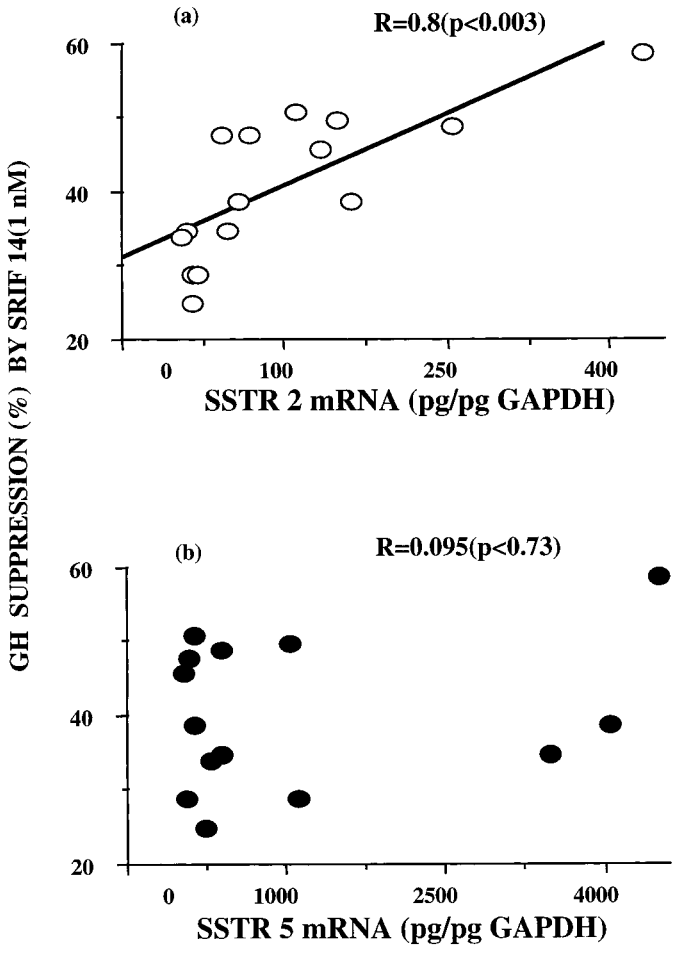

FIG. 3. Correlation scattergram between the GH suppression obtained in cell culture with 1 nmol/L SRIF14 and the level of mRNA expression for either SSTR2 (a) or SSTR5 (b).

Examples of the responses to the preferential SSTRs analogs are presented in Fig. 6. Among the 5 GH-only secreting adenomas, 3 showed similar GH suppression with either SSTR2- or SSTR5-preferring compounds, as exemplified by A1 (Fig. 6a), whereas in the remaining 2 GH-only secreting adenomas, GH was only suppressed by the SSTR2-preferential compound, BIM-23197 (A2, Fig. 6b). In these latter 2 tumors, GH suppression obtained with higher concentrations of BIM-23268 was interpreted as being due to the weak SSTR2 agonist activity of the compound. Among the 10 mixed adenoma cell cultures, 4 of 10 showed identical suppression of GH and PRL release with both the SSTR2- and the SSTR5-preferential compounds. In one such case (A6, Fig. 6c), the EC_{50} was 0.005 nmol/L for both GH and PRL suppression. In another 4 of 10 mixed adenomas a differential pattern of GH and PRL was observed in response to BIM-23197 and BIM-23268, as exemplified by A11 (Fig. 6d). The GH component of this tumor was highly sensitive to the inhibitory effect of the SSTR2-preferential agonist. Conversely, the PRL component was sensitive only to the inhibitory effects of the SSTR5-preferential agonist, BIM-23268. In this latter subgroup, such specific inhibitory effects of BIM-23197 and BIM-23268 on GH and PRL secretion argue for the coexistence of tumor subclones secreting either GH alone or PRL (or GH and PRL) alone. Finally, in 2 of 10 mixed adenomas the pattern of GH and PRL response to the two analogs was halfway between the 2 preceding examples, as GH suppression was highly sensitive to BIM-23197 only, whereas PRL was equally suppressed by BIM-23197 and BIM-23268. Thus, these individual data provide a more realistic overview of the functional heterogeneity among acromegalic tumors with regard to their sensitivity to the two SRIF analogs preferential for the SSTR2 and SSTR5 subtypes.

Additivity between the SSTR2- and SSTR5-preferential compounds in adenomas partially responsive to octreotide

The possibility of additivity of BIM-23197 and BIM-23268 was examined in three mixed GH+PRL tumors (A13, 14, and 15), which (*in vivo* studies) presented with a 18–40% partial inhibition of GH release after octreotide acute testing (Table 2 and Fig. 7). In these experiments the effects of BIM-23197 and BIM-23268, either alone or in combination, as well as of octreotide on GH and PRL secretion were examined using 10^{-12}-10^{-9} mol/L of each compound (and of equimolar concentrations when BIM-23197 and BIM-23268 were combined). The mean maximal GH suppression achieved by BIM-23197 plus BIM-23268 was 34 ± 6%, which was not statistically different from that obtained with BIM-23197 alone (25 ± 3%) or BIM-23268 alone (22 ± 7%). The maximal GH suppression produced by octreotide (14 + 7%) was significantly lower than that obtained with the combination of BIM-23197 and BIM-23268 ($P < 0.02$). These data indicate a lack of synergism between both compounds at maximally effective concentrations. Nevertheless, at submaximal concentration (10^{-10} mol/L), BIM-23197 plus BIM-23268 achieved a better GH and PRL inhibition than that obtained with either drug alone ($P < 0.05$). These data show that, in those tumors characterized as partial responders to octreotide, the combination of BIM-23197 and BIM-23268 is clearly additive within the dose-response range. In such tumors, the combination of SSTR2- and SSTR5-preferential compounds allowed comparable inhibition of GH and PRL with concentrations 10–30 times lower than those required when each drug was tested alone.

In situ hybridization studies (Fig. 8)

The cellular localization of SSTR2 and SSTR5 mRNA was analyzed on fragments of mixed tumors characterized as either sensitive (A6) or partially responsive (A15) to SRIF agonists (Table 2). In both tumors, hybridization with both PRL mRNA and GH mRNA antisense probes allowed identification of distinct hormonal cell phenotypes. About 18% of cells in both tumors were not labeled for either GH or PRL mRNAs. The cells expressing PRL mRNA only were extremely rare (4.7–1.3%, respectively, in each tumor). Based on the presence of cells labeled with either GH mRNA alone or with both GH and PRL mRNAs, both tumors were found to be bimorphous. In A6, the GH transcript alone was identified in 40% of the cells, whereas both GH and PRL transcripts were present in 41% of the cells. In A15, 64% of the cells expressed GH mRNA alone, whereas 15% expressed both GH and PRL transcripts. SSTR5 mRNA expression was observed in most of the cells from both tumors in both the pure

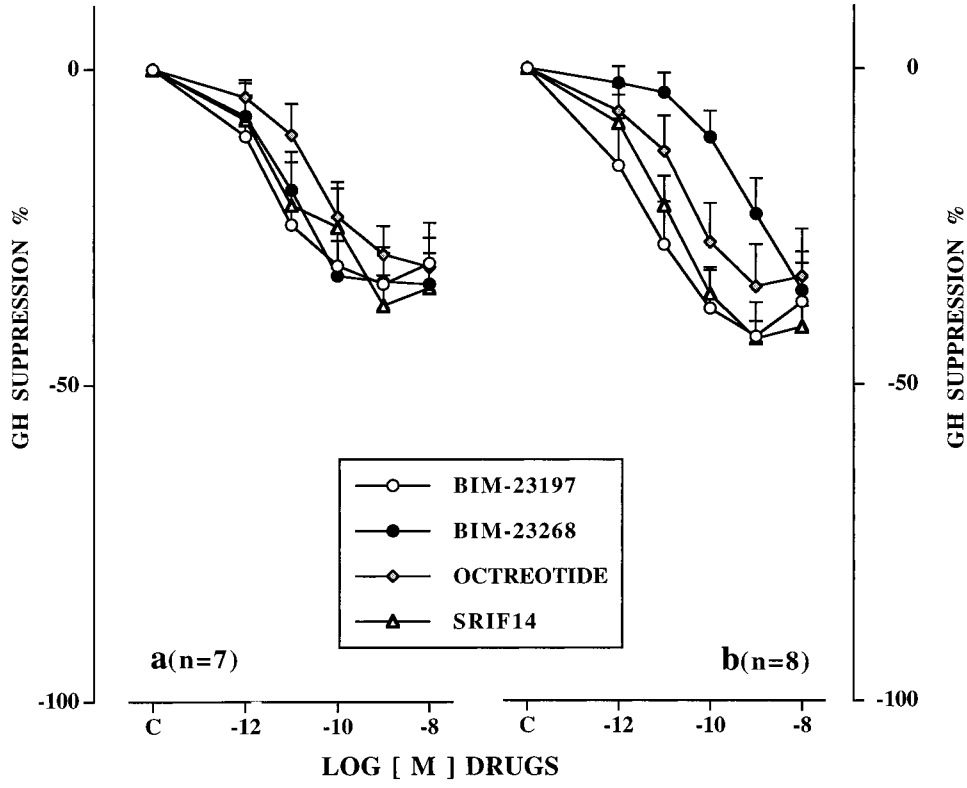

FIG. 4. Mean dose-response GH suppression curves obtained with SRIF14, BIM-23197, BIM-23268, and octreotide (10^{-12}–10^{-8} mol/L). Results are expressed as the mean ± SEM percent GH suppression *vs.* that with medium alone (control) in seven (group a) or eight (group b) somatotroph adenomas subclassified by the GH-suppressive effect of BIM-23268.

TABLE 3. Human somatostatin receptor subtype specificity

Compound	SSTR binding affinity (IC_{50}, nM)				
	1	2	3	4	5
Somatostatin-14	1.95	0.25	1.2	1.77	1.41
Somatostatin-28	1.86	0.31	1.3	ND	0.4
Octreotide	1140	0.56	34.5	7030	7
BIM-23197	6016	0.19	26.8	3897	9.8
BIM-23268	12	28	5.5	36	0.42

Data from radioligand receptor binding assays to membranes from transfected CHO-K1 cells expressing the different hSSTR subtypes. Values are from Biomesure, Inc. (personal communication) and Shimon *et al.* (13). ND, Not determined.

GH cells and the GH+PRL cells. The mean percentages of cells labeled with the SSTR5 probe were 85% and 87% for A6 and A15 tumors, respectively. With regard to SSTR2 mRNA labeling, a marked difference was observed between the two tumors. In the SRIF agonist-sensitive adenoma (A6), the SSTR2 transcript was expressed in 66–60% of the GH alone and GH+PRL cells. In the partially sensitive tumor (A15), SSTR2 mRNA expression was much lower. Only 29–30% of the GH alone and GH+PRL cells expressed the SSTR2 transcript. Interestingly the percentage of SSTR2 mRNA labeling (63% and 30%) in these two tumors correlated well with the 51% and 25% maximal inhibitory effects of SRIF14 on GH release observed in the cell culture studies of cells from the same adenomas (Fig. 2).

Discussion

Since 1985, SRIF binding and receptor autoradiographic techniques have revealed the presence of SSTRs in GH-secreting human pituitary tumors (2–4). The variable density of these receptors correlated with the variable GH response to octreotide in acromegalic patients (4). The more recent identification of the 5 human SSTR subtypes has led to the search for tissue- and tumor-specific patterns of subtype expression (16–18). Numerous qualitative studies, using RT-PCR, ribonuclease protection assay, or *in situ* hybridization techniques, have been performed in human pituitary GH-secreting adenomas (19–25). In these studies examining 65 different acromegalic adenomas, SSTR2 and SSTR5 transcripts were almost always identified (93% and 74% of the tumors, respectively). According to these previous studies, a highly variable expression of SSTR1 and SSTR3 was found (in 50% and 44% of the tumors, respectively). The SSTR4 transcript has never been observed in any of the adenomas. Our present quantitative RT-PCR analysis indeed confirms the constant coexpression of SSTR2 and SSTR5 mRNAs in adenomas from acromegalic patients. Despite individual variations, the relative expression of SSTR2 and -5 was similar in the tumors secreting GH alone or both GH and PRL. The mean level of SSTR5 mRNA expression was consistently much higher than that of the SSTR2 transcript. A preferential expression of the SSTR5 transcript has previously been reported, compared with the SSTR2 transcript, in 10 GH or

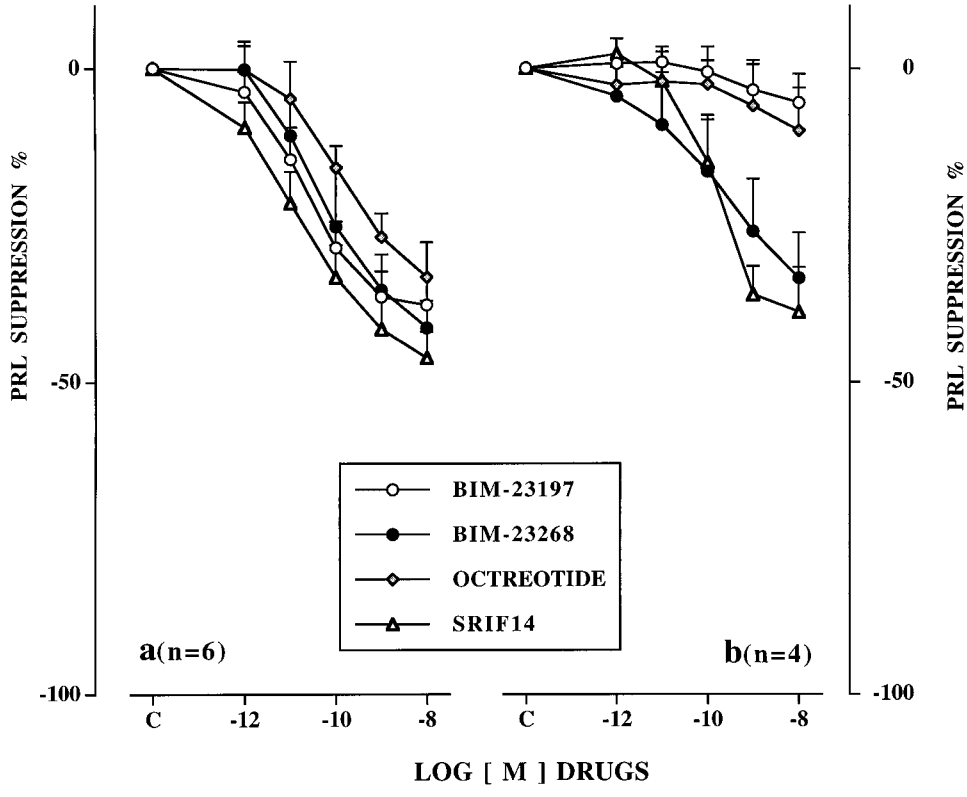

FIG. 5. Mean dose-response PRL suppression curves with SRIF14, BIM-23197, BIM-23268, and octreotide (10^{-12}-10^{-8} mol/L). Results are expressed as the mean ± SEM percent GH suppression *vs.* that with medium alone (control) in six (group a) or four (group b) GH+PRL mixed adenomas subclassified by the PRL-suppressive effect of BIM-23197.

GH+PRL adenomas (20). Regarding the quantitative expression of the other SSTR transcripts, we also observed variable expressions of SSTR1 and SSTR3 mRNAs, whereas SSTR4 mRNA was never observed among the 15 tumors. Our data concerning SSTR1 mRNA expression provide an explanation for the discrepancies previously observed in the literature. The SSTR1 transcript was poorly expressed among the pure GH adenomas. It was essentially expressed, to varying degrees, in the mixed GH+PRL adenomas. Such an association of SSTR1 mRNA to the lactotroph component of the acromegalic tumors is supportive of the qualitative and quantitative observations of SSTR1 expression already reported in human pure lactotroph pituitary adenomas (9, 19). The variable expression of SSTR3 mRNA, as observed in our tumors, has been previously discussed. The widespread expression of SSTR3 in non-GH-secreting pituitary tumors suggests its involvement in functions other than the regulation of GH or PRL secretion (20). Finally, the absence of SSTR4 mRNA expression in our tumors agrees with recent data showing that SSTR4 was the least expressed subtype in the rat pituitary, detectable in only a few of the somatotrophs (26). Thus, the predominant SSTR2+SSTR5 pattern of mRNA expression consistently observed in both pure and mixed somatotroph tumors differs from the predominant SSTR5+SSTR1 pattern of expression observed in pure human lactotroph tumors (9). Recently, a similar difference in SSTR subtype expression was recognized in the different endocrine cells of the human pancreas (27), in which SSTR1-SSTR5 subtypes are expressed in the β-cells, SSTR2 is more selectively expressed in the α-cells, and SSTR5 is quasiselectively expressed in the δ-cells. Such cellular specificities for the different SSTR subtypes indeed underlines the need for SSTR-specific ligands to target hormone suppression.

The expression of SSTR2 mRNA was quantitatively correlated to the degree of GH release inhibition by both SRIF14 and SRIF28 in our study. No difference in the inhibition of GH release was observed with either native SRIF in most cases. The dominant expression of SSTR5 mRNA, however, was not correlated to either GH or PRL suppression by any of the compounds tested. Whether the observed high expression of SSTR5 mRNA correlates with the presence of SSTR5 protein is unknown. The expression of SSTR mRNAs has been correlated to the receptor proteins in some studies (18, 28), but not in others (29). Further studies, using specific antibodies raised against the different SSTR subtypes, are necessary to investigate whether there are either transcriptional defects or abnormal coupling mechanisms that may explain SSTR5 nonfunctionality in some GH-secreting tumors (30). The presence of the SSTR5 cannot be explained on the basis of its role in suppressing PRL secretion, as equivalent SSTR5 mRNA expressions were found in pure GH and mixed GH+PRL tumors.

Although SRIF14 and SRIF28 were equipotent in hormone suppression, the SSTR2 and SSTR5 preferential analogs identified heterogeneous suppressive effects on GH and PRL secretion. In the past, the subtype selectivity of some peptide SRIF analogs has been questioned (31). More recently, cyclic and linear SRIF analogs have been developed and present with a preferential selectivity for SSTR2, such as BIM-23197 (32), or for SSTR5, such as BIM-23268 (33). This latter compound has a 40-fold selectivity in affinity for SSTR5 over SSTR2 when tested for binding in CHO-1 cells transfected

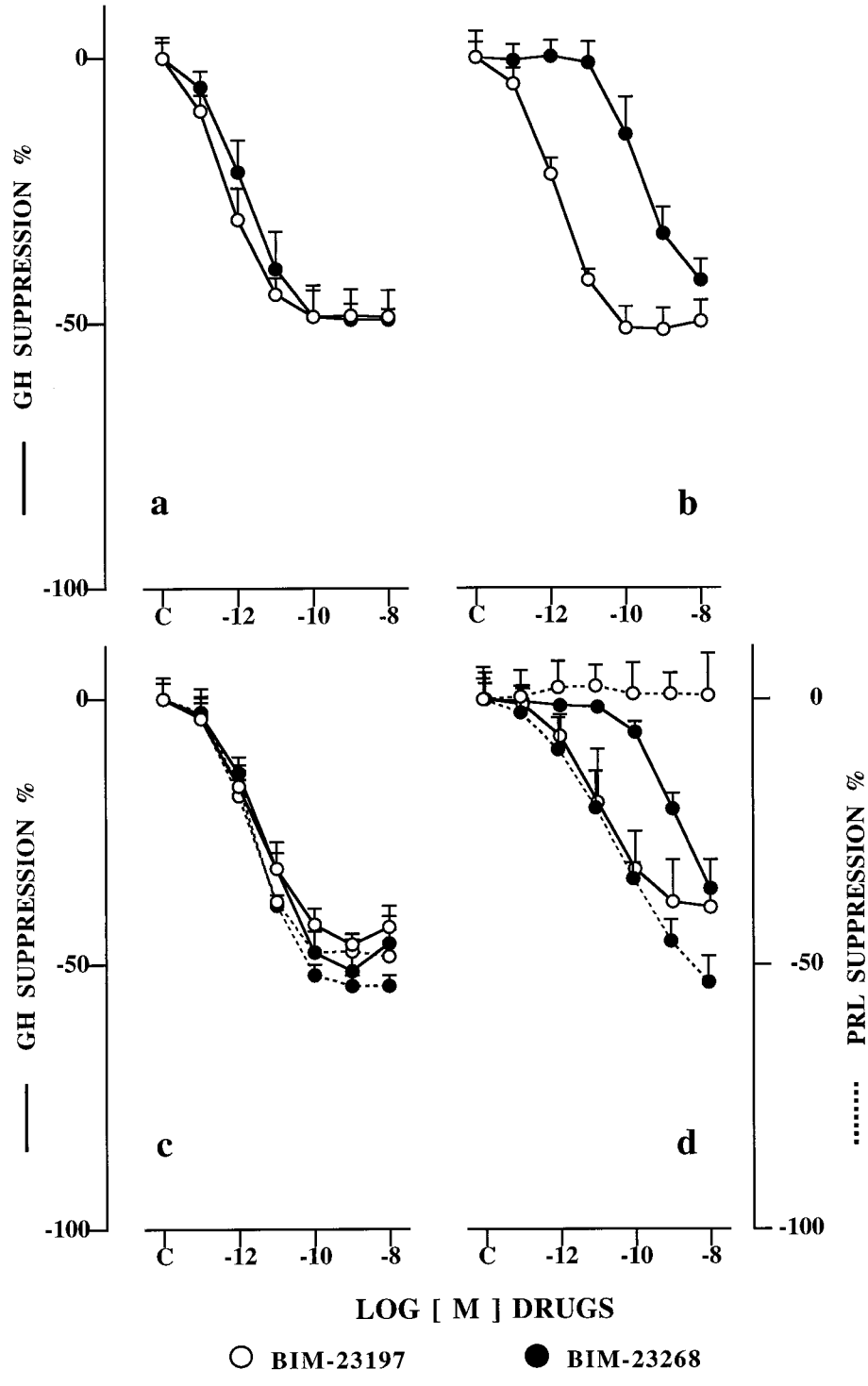

FIG. 6. Individual BIM-23197 *vs.* BIM-23268 dose-response curves (10^{-13}-10^{-8} mol/L) for the inhibition of GH (*solid line*) or PRL (*dotted line*) secretion in four distinct adenoma cell cultures. In a (A1) and b (A2), pure GH adenomas are shown; in c (A6) and d (A11), mixed GH+PRL adenomas are shown. Values are the percent GH or PRL suppression *vs.* the control value (medium alone) and are the mean ± SEM of four wells.

with the human receptor subtypes (33). Classification of the tumors on the basis of their hormonal response patterns to the SSTR2- and SSTR5-preferring analogs revealed distinct phenotypes that can be correlated with prior morpho-functional subclassifications of somatotroph adenomas (34). In somatotroph adenomas, the tumor cells present either as GH only, densely or sparsely granulated cells or as mixed GH+PRL adenoma cells (35). In the latter case, according to double labeling immunogold electron microscopy, the tumors appear either as monomorphous (GH and PRL granules colocalized in the same mammosomatotroph cell) or as bimorphous tumors composed of a mixture of both GH only and PRL only adenomas cells. In initial studies, the GH only adenomas were reported to represent 64% of the tumors (35). A more recent immunohistochemical analysis of 69 acromegalic tumors revealed PRL- as well as α-subunit-immunopositive cells in 90% of somatotroph tumors (36). The preponderance of mixed GH+PRL tumors was also encountered in

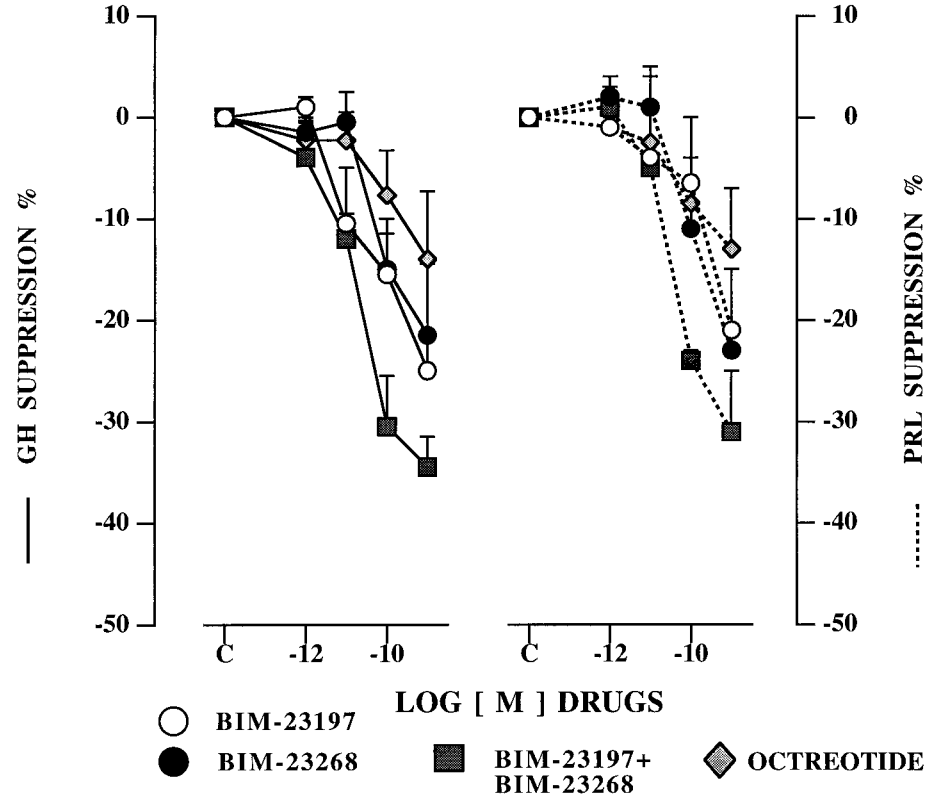

FIG. 7. Mean GH (*solid line*) or PRL (*dotted line*) suppression dose-response curves with BIM-23197, BIM-23268, octreotide, or BIM-23197 plus BIM-23268 (10^{-12}-10^{-9} mol/L). Results are expressed as the mean ± SEM percent GH or PRL suppression *vs.* medium alone (control) in three octreotide partially responsive GH+PRL mixed adenomas.

our *in vitro* study, which detected an associated mild PRL secretion in 66% of the adenoma cell cultures. Temporal analysis of the somatolactotrope lineage during fetal development displays several different morphological steps in somatomamotroph cell commitment (37) that are superimposable on the different ultrastructural aspects of the somatotroph adenomas. The evolving human fetal pituitary initially displays well differentiated somatotroph cells at 8–9 weeks. A second population of mammosomatotroph cells, appearing at 12 weeks gestation, develops during the second trimester of pregnancy. Finally, the pure lactotroph cells differentiate from week 23 to the term of gestation. This developmental progression is corroborated by the experimental model of transgenic mice bearing the thymidine kinase obliteration system (38). In keeping with the hypothesis that adenomas could occur from cells at any stage of somatolactotroph development, our analysis of the variable inhibitory effects of the SSTR-preferential compounds suggests an evolutive pattern for the differentiation of SSTR subtype functionality. The progenitor cell somatotroph adenoma could bear both SSTR2 and SSTR5 functional subtypes. These two SSTRs subtypes remain functional for GH and PRL inhibition in the mammosomatotroph bisecreting adenoma. This concept is supported by the results of a previous study of human fetal pituitary cells, collected at weeks 23–25 (*i.e.* when both somatotroph and somatomammotroph cells are well differentiated), in which both BIM-23197 and BIM-23268 were shown to equally inhibit GH release (13). The third tumoral cell phenotype arises from the somatomammotroph lineage in which there is somatostatinergic regulation with preferential GH suppression through SSTR2 and preferential PRL suppression through SSTR5. The fourth somatotroph tumor phenotype is characterized by its SSTR2-only regulation of GH suppression. It has been previously shown that the suppression of PRL in pure prolactinomas was mediated mainly via the SSTR5 subtype (8, 9), whereas the SSTR2-preferential agonists only occasionally could mediate PRL suppression (9). Such arguments favor an ultimate differentiation in which some lactotrophs lose the SSTR2 regulatory component, as opposed to the terminal differentiation of somatotrophs, in which the SSTR5 regulatory component is lost. Such a differentiation pathway from progenitor cells, as proposed in the theories of stem cell regulation (39), implies a default of differentiation executed by the stem cell under factors influencing the pituitary cell commitment. This was already documented for the GHRH receptor (40), which plays an important role in the differentiation of somatotrophs. The suggestion of an evolutive pattern of expression of SSTRs subtypes is not surprising, as it has already been documented during the ontogenesis of the fetal rat brain, which initially expresses SSTR2 and SSTR3 in different areas, whereas SSTR4-SSTR5 emerge only around birth (41).

Finally, and possibly most importantly from a therapeutic perspective, our study as well as previous cell culture studies of acromegalic tumors (8, 42–45) underline the variable suppressive effects of SRIF agonists on GH secretion, according to the individual nature of each tumor. In the adenoma cells taken from acromegalic patients that were partially responsive to the inhibitory effects of octreotide, our hybridization studies revealed a clear loss of SSTR2 transcript expression in the majority of the adenoma cells. Such findings underline the key role of the SSTR2 subtype in the inhibitory regulation

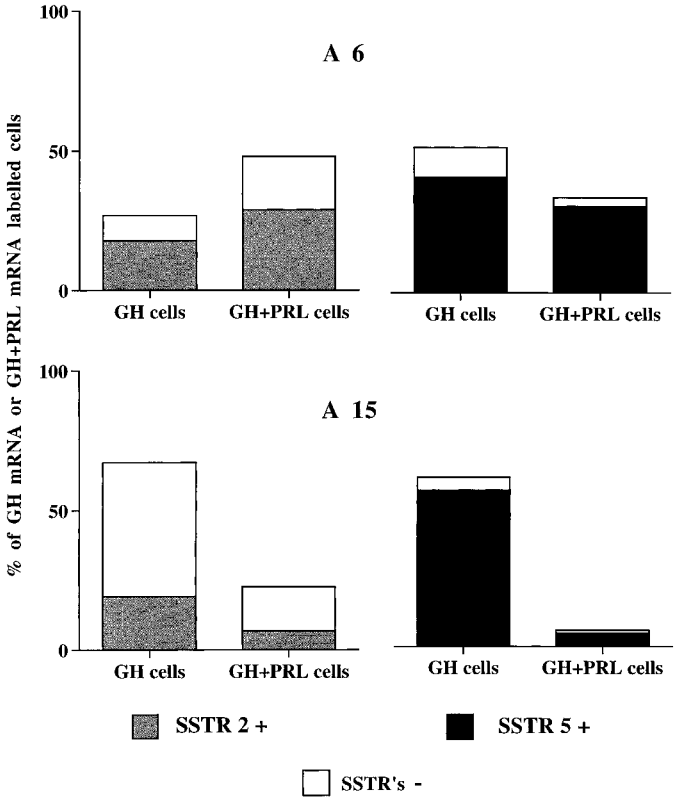

FIG. 8. Percentage of GH or GH+PRL mRNA-labeled cells expressing SSTR2 or SSTR5 mRNAs in two mixed adenomas, either sensitive (A6) or partially responsive to octreotide (A15). Cell counts ranged from 2644–3544 cells. Unlabeled cells (18%) and PRL-only labeled cells (1.3–4.7%) are not represented.

of GH release. Identical observations of a patchy, heterogeneous distribution of SSTR2 in GH-secreting tumors, poorly responsive to octreotide administration, have been previously noticed by receptor autoradiography in two tumors (4). However, it should be noted that in addition to the SSTR2 level of expression, other mechanisms are involved in the sensitivity of GH-secreting adenomas to SRIF analogs. In fact, despite a better octreotide sensitivity of gsp^+ *vs.* gsp^- adenomas, SSTR2 mRNA is not different in the two categories of tumors (46). In the GH-secreting tumors partially responsive to octreotide or lanreotide, can we expect an additive suppressive effect on GH secretion when the SSTR2 and SSTR5 analogs are combined? Indeed, in a previous report of three cultured octreotide- or lanreotide-resistant adenomas (8), the combination of both BIM-23197 and BIM-23268 was found to significantly improve the inhibition of GH. Such additivity was nevertheless partial, as an only a 10% greater inhibitory effect was obtained by the combination of both compounds compared with the maximal GH inhibition achieved by one of the drugs alone. In our three cases, combining SSTR2- and SSTR5-preferential analogs did not produce a significant synergistic effect in suppressing GH. Nevertheless, at submaximal concentrations, a partial additivity of BIM-23197 and BIM-23268 on GH suppression was observed. Such data can be explained by the fact that such a drug combination results in binding affinities of 0.19 and 0.42 nmol/L, respectively, for the SSTR2 and SSTR5 subtypes, which are similar to those of native SRIF28. Furthermore, in our studies, both BIM-23197 and 23268 produced a greater maximal PRL suppression than that obtained using octreotide. Due to the variable tumor cell phenotypes and to the variable distribution of SSTR2 and SSTR5 transcripts, the combination of both analogs may mimic the effects of the native SRIF more accurately than octreotide or lanreotide, and could allow better control of hormone hypersecretion in acromegalic tumors.

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