

Effects of Octreotide Treatment on the Proliferation and Apoptotic Index of GH-Secreting Pituitary Adenomas

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To investigate the effects of octreotide administration on the growth rate of GH-secreting pituitary adenomas, we measured both the Ki-67 labeling index (LI) and the apoptotic index in tumor specimens from octreotide-treated or matched untreated acromegalic patients. Thirty-nine patients who received octreotide until the day of or the day before surgery and 39 untreated patients matched for sex, age, tumor size, extension, and invasiveness were studied. Immunocytochemical analysis was performed on paraffin-embedded material using a monoclonal antibody (MIB-1) directed against a proliferation-associated nuclear antigen, Ki-67, to measure the growth fraction. Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end-labeling method, using a monoclonal antibody recognizing areas of DNA fragmentation. The Ki-67 LI and apoptosis were counted on separate slides in at least 1000 evaluable cells.

Octreotide-treated patients showed a lower Ki-67 LI ($1.8 \pm$

0.3%) than untreated controls ($3.8 \pm 0.7\%$; $P < 0.02$). Overall, the mean Ki-67 LI of treated patients was 53% lower than that in untreated patients. The antiproliferative effect of octreotide occurred independently of tumor extension and invasiveness. Octreotide-treated and untreated patients showed similar apoptotic indexes ($0.6 \pm 0.2\%$ and $0.8 \pm 0.3\%$, respectively). There was a positive correlation between the Ki-67 LI and the apoptotic index ($r = 0.29$; $P < 0.03$).

Our study demonstrates that acromegalic patients receiving chronic octreotide treatment have a lower value of the proliferation marker Ki-67, but no significant difference in the apoptotic index compared with matched untreated patients. The antiproliferative effect of octreotide on GH-secreting adenomas should imply a lower risk of tumor growth during long-term chronic treatment with the drug. (*J Clin Endocrinol Metab* 86: 5194–5200, 2001)

SOMATOSTATIN PLAYS AN important role in the regulation of GH secretion in humans. The effect of somatostatin is mediated through high affinity, G protein-coupled membrane receptors that are expressed in all somatostatin-target organ systems. Five somatostatin receptor subtypes have been cloned and characterized (1, 2). The five somatostatin receptors have a tissue-specific distribution and the majority of responsive tissues express multiple somatostatin receptor subtypes (3). In contrast to native somatostatin, which binds to all five somatostatin receptors with high affinity, the long-acting somatostatin analogs, octreotide, lanreotide, and vapreotide, bind with high affinity to somatostatin receptor subtype 2, with lower affinity to subtypes 3 and 5, and have no affinity to subtypes 1 and 4 (4).

Octreotide was introduced in the medical treatment of active acromegaly about 15 yr ago and quickly gained widespread acceptance as a secondary as well as a primary therapy because of its efficacy and tolerability (5, 6). Octreotide not only inhibits GH secretion, but also reduces tumor size in 30–40% of acromegalic patients (7–9), although the degree of tumor shrinkage is only marginal in most cases. Studies reporting the morphological effects of chronic treatment with

octreotide on GH-secreting adenomas have yielded conflicting results (10–14). Degenerative changes in treated tumors, such as capillary reduction, fibrosis, and necrosis, were noted by most researchers (10, 12, 13). However, the most striking observation was the marked heterogeneity of morphological changes (14), which might be explained by the well known different expression of functional somatostatin receptors among various GH-secreting adenomas and even within a single tumor (15).

The question of whether octreotide treatment exerts an antiproliferative effect on GH-secreting adenomas has not been thoroughly investigated. The rate of growth of the pituitary tumor depends on the balance between the proliferating cells and the loss of tumor cells by apoptosis (programmed cell death) and ischemic or hemorrhagic events (16, 17). Few studies investigated this issue in GH-secreting adenomas; octreotide-treated acromegalic patients showed a significant reduction of the Ki-67 labeling index (LI) compared with untreated patients (18, 19), whereas no difference in the apoptotic index was detected in similarly treated patients (20, 21). However, no study reported the results of both proliferation and apoptotic indexes in the same tumors.

With the aim to verify the existence of an antiproliferative effect of octreotide treatment in acromegalic patients, we compared the Ki-67 LI and apoptotic index of GH-secreting

Abbreviations: LI, Labeling index; MRI, magnetic resonance imaging; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling.

adenomas from patients treated with octreotide until surgery or from matched untreated patients.

Subjects and Methods

Patients

Eligible patients were all patients with active acromegaly operated on between 1990 and 1999 in one of the two participating centers [San Raffaele Milan, Italy and Centro Traumatologico Ortopedico (Turin)] who were receiving treatment with octreotide until the day of or the day before surgery. Minimal duration of octreotide therapy for inclusion into the study was 2 months at a daily dose of at least 0.2 mg, sc. Diagnosis of active acromegaly was based on the clinical picture, demonstration of GH hypersecretion (usually confirmed by the failure of serum GH to suppress below 2 $\mu\text{g}/\text{liter}$ during an oral glucose tolerance test and elevated levels of IGF-I), and a pituitary tumor demonstrated on magnetic resonance imaging (MRI). Gonadal status in premenopausal women was defined according to menstrual status. Postmenopausal women were considered hypogonadal unless they were receiving E replacement therapy. Men were classified according to their basal T level. Pituitary tumor size was estimated by measuring the maximum anteroposterior, vertical, and horizontal diameters on MRI. A reduction of at least 20% of any tumor diameter on a posttreatment examination, if available, was considered significant.

Untreated patients were randomly selected from all of the other acromegalic patients operated on in the same period who had not received octreotide or other somatostatin analogs. Untreated patients were matched for age (within 10 yr), sex, tumor size (defined as micro- or macroadenoma), extrasellar extension of the tumor (yes or no), and invasion of the cavernous sinus on the preoperative MRI (yes or no). When more than one untreated patient matched a case patient, the one operated nearest to the case patient was selected for the study. Classification of tumor characteristics for octreotide-treated patients was based on the pretreatment MRI scan.

The following exclusion criteria were applied to both octreotide-treated and untreated patients: 1) previous pituitary surgery, 2) previous pituitary radiotherapy, and 3) clinical and histological diagnosis of pituitary apoplexy. Moreover, untreated patients were not eligible for the study if they had received treatment with octreotide or another somatostatin analog, even when therapy had been stopped months or years before pituitary surgery.

Histological analysis

Surgically removed specimens were immediately fixed in 10% buffered formalin and subsequently embedded in paraffin. Standard hematoxylin-eosin sections were used for diagnosis. Analysis of MIB-1 immunostaining and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL; see below) was conducted on tumoral tissue from the same paraffin-embedded block used for immunocytochemical analysis.

MIB-1 immunostaining

Proliferation activity of the tumor was determined by calculating the percentage of cells exhibiting the Ki-67 antigen, a nuclear protein of unknown function expressed only in cycling cells (17). To this aim we used the monoclonal antibody MIB-1 (Immunotech, Marseilles, France), which reacts with an epitope encoded by a 1002-bp fragment of the Ki-67 cDNA (22). At variance with other methods, MIB-1 antibody is suitable for use in paraffin-fixed material. Technical details have been previously described (23). Briefly, MIB-1 immunostaining was performed on 4- μm sections mounted on glass slides treated with poly-L-lysine and dried until ready for use. Tissue sections were dewaxed, and endogenous peroxidase activity was blocked. Then, after antigen retrieval by microwave treatment (two passages at 850 watts of 5 min each), sections were incubated with nonimmune rabbit serum at room temperature for 20 min to block nonspecific links and then incubated at 37 C for 60 min with MIB-1 monoclonal antibody diluted 1:50 (Immunotech); no MIB-1 antibody was added to sections used as negative control samples. After rinsing, slides were incubated with rabbit biotinylated antimouse IgG (diluted 1:100) as secondary antibody for 30 min at room temperature

and then exposed to streptavidin-biotin-peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) for 30 min. After rinsing, sections were exposed in the dark for 5 min to the chromogen 3,3'-diaminobenzidine, which stains Ki-67-immunopositive nuclei with a dark brown color. Slides were lightly counterstained with hematoxylin, dehydrated, cleared, and mounted. Because MIB-1-positive cells may be unevenly distributed in biopsy samples, the Ki-67 LI was determined by counting a total of at least 1000 neoplastic nuclei subdivided in 10 randomly chosen fields at $\times 400$ magnification. Sections of human cecal appendix were used as positive control samples.

Determination of apoptosis

The TUNEL method was used after minor modifications, as previously described (23). Briefly, tissue sections of 4 μm were mounted on glass slides treated with poly-L-lysine, deparaffinized, hydrated, and treated for 10 min at 37 C with proteinase K (Roche, Mannheim, Germany; 20 $\mu\text{g}/\text{ml}$ 10 mM Tris-HCl buffer, pH 7.4). Slides were rinsed twice with PBS. Then, 50 μl TUNEL reaction mixture (450 μl nucleotide mixture containing fluoresceinated deoxy-UTP in reaction buffer plus 50 μl enzyme terminal deoxynucleotidyl transferase from calf thymus) were added to samples. Slides were incubated in a humidified chamber for 60 min at 37 C. After rinsing, slides were incubated with antifluorescein antibody, Fab from sheep, conjugated to alkaline phosphatase for 30 min at room temperature (Roche). Then, on each slide, 50 μl substrate solution (blue tetrazolium) were added, and slides were incubated for 10 min at room temperature. Positive signal was defined as the presence of a distinct blue staining on nuclei of the neoplastic cells or on apoptotic bodies as morphologically defined. The apoptotic index was determined by counting a total of at least 1000 neoplastic nuclei subdivided into 10 randomly chosen fields at $\times 400$ magnification. Apoptotic cells were identified by TUNEL in conjunction with characteristic morphological changes, such as cell shrinkage, membrane blebbing, and chromatin condensation, to distinguish apoptotic cells and apoptotic bodies from necrotic cells. The latter were not considered apoptotic cells. Sections of human cecal appendix were used as positive controls.

All Ki-67 LI and apoptotic index determinations were performed by a single pathologist (F.M.), who was unaware of the clinical characteristics and treatment assignments of the patients.

Hormone assay

Serum GH levels were measured by commercially available immunofluorimetric assays (Tosoh, Tokyo, Japan). Serum PRL levels were determined by a commercially available immunoenzymatic assay (Immuno 1, Bayer Corp., Divisione Diagnostici, Milan, Italy). Serum IGF-I was measured by a specific commercially available RIA after acid extraction (Bioclone, Marrickville, Australia).

Statistical analysis

All data are expressed as the mean \pm SE. Because the distributions of Ki-67 LI, apoptotic index, and GH levels both at presentation and during octreotide treatment were markedly skewed, we performed logarithmic transformation of the data; a value of 0% for apoptotic index was arbitrarily assigned to be 0.05%. However, Ki-67 LI, apoptosis index, and GH levels are presented in the usual decimal format in the text and figures. A *t* test for unpaired or paired data, as appropriate, was used to compare continuous variables among groups. Correlation between continuous variables was calculated by the Spearman rank correlation coefficient method. χ^2 tabulation was used to compare binomial proportions. A two-factor ANOVA was performed to evaluate the interaction between selected tumor characteristics and treatment group on the proliferation and apoptotic indexes. *P* < 0.05 was considered to indicate statistical significance. All calculations were performed using the statistical package StatView 4.0 (Abacus Concepts, Inc., Berkeley, CA).

Results

Patients' characteristics

We analyzed the data from 78 acromegalic patients. All operations were performed through the transsphenoidal

TABLE 1. Clinical and hormonal characteristics of 78 acromegalic patients according to preoperative treatment with octreotide

Patients' characteristics	Octreotide (n = 39)	No treatment (n = 39)
Sex (F/M)	25/14	25/14
Age (yr)	40.9 ± 1.9	41.4 ± 2.0
Yr of surgery (median)	1995	1995
Previous dopaminergic therapy (yes/no)	7/32	7/32
Tumor size (micro/macro)	2/37	2/37
Extrasellar extension (yes/no)	19/20	19/20
Maximum tumor diameter (mm) ^a	21.8 ± 1.7	20.8 ± 1.6
Invasion of cavernous sinus (yes/no)	8/31	8/31
Basal GH (μg/liter)	38.7 ± 6.1	43.2 ± 16.9
Basal PRL (μg/liter)	17.1 ± 2.8	24.9 ± 8.3
Hypogonadism (yes/no) ^b	19/17	25/13

There was no significant difference between octreotide-treated and untreated patients in any of the clinical and hormonal characteristics.

^a MRI measurement of maximum tumor diameter was available for 31 pairs of case and control patients.

^b Information about gonadal status was not available in three octreotide-treated patients and in one untreated patient.

approach. Clinical and demographic characteristics of octreotide-treated and untreated patients are summarized in Table 1. As expected, the two groups of patients, treated and untreated, were similar with regard to sex distribution, mean age at surgery, tumor size, tumor extension, and tumor invasiveness into the cavernous sinus. Moreover, there were no significant differences between the two groups in other characteristics that had not been used for matching, such as year of surgery, mean basal GH and PRL levels at presentation, maximum tumor diameter, history of previous dopaminergic therapy, and gonadal status at surgery (Table 1). Dopamine agonist drugs were continued until 3 months before surgery in eight patients (five octreotide-treated and three untreated), whereas in the remaining six patients dopaminergic treatment was stopped 3–36 months before surgery. Exclusion of patients who received dopaminergic drugs within 3 months from surgery and their relative matches did not significantly alter any results.

Octreotide-treated patients received the drug for a mean period of 12.1 ± 1.9 months before surgery, ranging from 2–62 months. The daily drug dose was 0.356 ± 0.023 mg, ranging from 0.2–0.9 mg. The total amount of octreotide received by the patients before surgery, calculated as the mean daily dose of the drug multiplied by 30 and then by the months of treatment, ranged from 18–630 mg, with a mean value of 137 ± 24 mg. The mean basal GH level decreased from 38.7 ± 6.1 to 19.1 ± 3.4 μg/liter ($P < 0.001$) during octreotide treatment in the 34 patients for whom both values were available. Reduction of the basal GH level more than 50% of the pretreatment value occurred in 22 of the 34 patients (65%), and 6 of them (17.6%) reached a basal GH level lower than 2.5 μg/liter. Serum IGF-I levels before and during octreotide treatment were available for 17 patients only. IGF-I levels decreased from a mean pretreatment value of 924.1 ± 74.0 to 595.8 ± 45.0 μg/liter ($P < 0.001$) during octreotide treatment, but normalized in only 1 patient. Pre- and posttreatment MRI scans were available for review in 25

patients. Reduction of at least 20% of any tumor diameter occurred in 5 cases (20%).

Ki-67 LI

The mean Ki-67 LI in both treated and untreated patients was 2.8 ± 0.4%, ranging from 0.1–18.8% (median, 1.6%). Octreotide-treated patients showed a lower Ki-67 LI (1.8 ± 0.3%) than untreated patients (3.8 ± 0.7%; $P < 0.02$; Fig. 1). Overall, the mean Ki-67 LI of treated patients was suppressed by 53% compared with that in untreated patients.

Tumors with extrasellar extension had a higher Ki-67 LI (4.0 ± 0.7%) than intrasellar tumors (1.7 ± 0.3%; $P < 0.001$), and a similar, but weaker, relationship was observed for invasiveness into the cavernous sinus (4.6 ± 1.2% in invasive tumors vs. 2.4 ± 0.4% in noninvasive tumors; $P < 0.03$), whereas sex, age, history of previous dopaminergic therapy, year of surgery, preoperative GH level, and center had no significant effect on Ki-67 LI. To assess the relationships among octreotide pretreatment, Ki-67 LI, and tumor extension and invasiveness, we performed a two-way ANOVA. Both octreotide treatment and tumor extension affected Ki-67 LI ($F = 7.2$; $P < 0.01$ and $F = 15.6$; $P < 0.001$, respectively; Fig. 2), but there was no significant interaction ($F = 1.1$; $P = 0.30$). Octreotide treatment and tumor invasiveness had an effect on Ki-67 LI ($F = 5.0$; $P < 0.03$ and $F = 5.4$; $P < 0.03$, respectively; Fig. 2), but again no significant interaction was observed ($F = 0.2$; $P = 0.70$). These results indicate that the effects of octreotide on the Ki-67 LI occurred independently of tumor extension and invasiveness. Gonadal status at the time of surgery did not significantly affect the Ki-67 LI val-

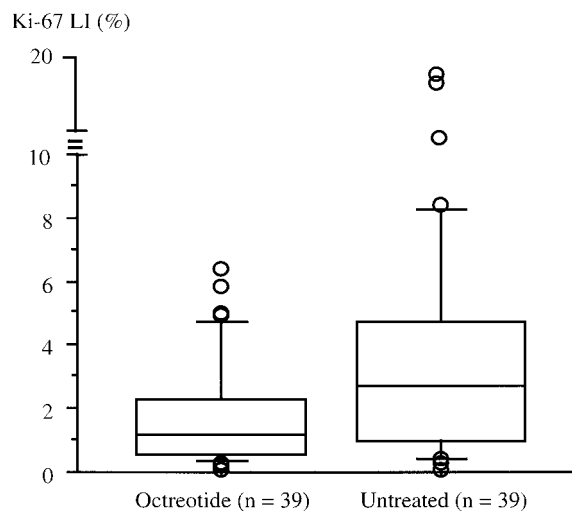


FIG. 1. Box graphs and whisker plots representing the distribution of Ki-67 LI in 78 GH-secreting pituitary adenomas exposed and unexposed to preoperative octreotide therapy. The box contains values between the upper and lower quartiles and shows the interquartile range (IQR). The line cutting the box is the median. The lines, or whiskers, extend from the ends of the box to either the largest values that are within the range upper quartile + 1.5 × IQR or to the smallest values within the range lower quartile - 1.5 × IQR. More extreme values are shown as individual points. Note the break on the y-axis. Because of the skewed distribution, the data were log-transformed before statistical analysis. The mean Ki-67 LI of treated patients was 53% lower than that in matched untreated patients. The difference between the two groups is significant ($P < 0.02$).

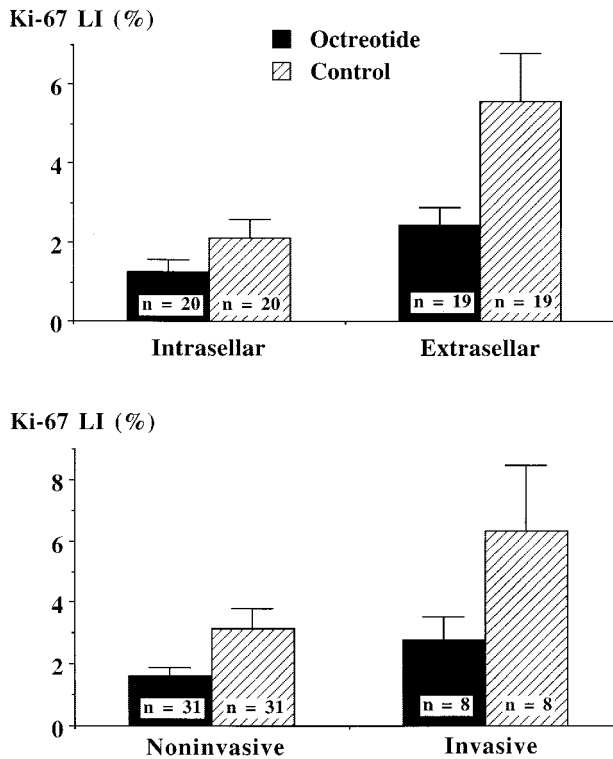


FIG. 2. Mean \pm SE Ki-67 LI in 39 octreotide-treated patients and 39 untreated patients stratified by tumor extension (intracellular vs. extrasellar; upper panel) and tumor invasiveness into cavernous sinus (noninvasive vs. invasive; lower panel). Octreotide treatment was associated with a significantly lower Ki-67 LI in each subgroup considered.

ues. Overall, the 44 hypogonadal patients had a Ki-67 LI ($2.8 \pm 0.6\%$) similar to that of the 30 eugonadal patients ($2.9 \pm 0.5\%$). A 2-way ANOVA did not detect any significant interaction between octreotide treatment and gonadal status on the Ki-67 LI.

The duration of preoperative octreotide therapy and the total amount of octreotide administered were not correlated with the Ki-67 LI (Spearman correlation coefficient = 0.07 and 0.19, respectively). There was no significant difference in Ki-67 LI between the group of 22 patients in whom octreotide therapy inhibited GH secretion by more than 50% ($1.4 \pm 0.3\%$) compared with the other 12 patients who did not have such a reduction ($2.0 \pm 0.6\%$). However, the 6 patients who reached a basal GH level below $2.5 \mu\text{g}/\text{liter}$ during octreotide therapy had a Ki-67 LI ($0.6 \pm 0.1\%$) significantly lower than that of patients whose basal GH level remained higher than $2.5 \mu\text{g}/\text{liter}$ ($2.2 \pm 0.4\%$; $P < 0.01$). Tumors that shrunk during octreotide treatment had a significantly lower Ki-67 LI ($0.6 \pm 0.2\%$) than tumors whose size remained unchanged ($2.1 \pm 0.4\%$; $P < 0.02$).

Apoptosis

Apoptosis could not be evaluated in 7 samples (4 from case patients and 3 from control patients) because of technical failure. In the following analyses we also excluded the respective matched untreated or octreotide-treated patients. Therefore, the data for apoptosis pertain to 32 pairs of patients.

The mean apoptotic index was $0.7 \pm 0.2\%$, ranging from 0–8.7% (median, 0.4%). Octreotide-treated and untreated patients showed similar apoptotic indexes ($0.6 \pm 0.2\%$ and $0.8 \pm 0.3\%$, respectively; Fig. 3). Sex, age, history of previous dopaminergic therapy, year of surgery, extrasellar extension of the tumor, preoperative GH level, and center did not significantly affect the apoptotic index in the whole group of patients. On the contrary, patients with tumor invading the cavernous sinus had a higher apoptotic index ($1.5 \pm 0.6\%$) than patients with noninvasive tumors ($0.5 \pm 0.1\%$; $P < 0.05$). In the 32 pairs of evaluable patients, there was a positive correlation between the Ki-67 LI and the apoptotic index (Spearman correlation coefficient = 0.29; $P < 0.03$). Apoptotic index was not influenced by the gonadal status ($0.7 \pm 0.2\%$ in hypogonadal patients vs. $0.9 \pm 0.3\%$ in eugonadal patients). No significant interaction between octreotide treatment and gonadal status on the apoptotic index was detected.

Duration of preoperative octreotide therapy and total amount of octreotide administered showed a borderline correlation with the apoptotic index (Spearman correlation coefficient = 0.32 and 0.29; $P = 0.06$ and 0.10, respectively). The apoptotic index was not significantly lower in the 21 patients in whom octreotide therapy inhibited GH secretion by more than 50% ($0.5 \pm 0.1\%$) compared with the other 9 patients who did not have such a reduction (1.0 ± 0.5). A similar result was obtained when we subdivided the patients according to whether they had reached a basal GH value below $2.5 \mu\text{g}/\text{liter}$ during octreotide therapy. The apoptotic index in the group of patients whose tumor shrunk during octreotide therapy was not significantly different from that in the group

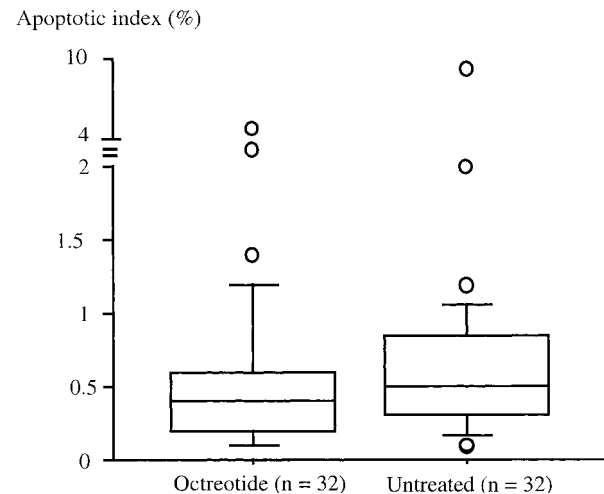


FIG. 3. Box graphs and whisker plots representing the distribution of the apoptotic index in 64 GH-secreting pituitary adenomas exposed and unexposed to octreotide therapy preoperatively. The box contains values between the upper and lower quartiles and shows the interquartile range (IQR). The line cutting the box is the median. The lines, or whiskers, extend from the ends of the box to either the largest values that are within the range upper quartile $+1.5 \times$ IQR or to the smallest values within the range lower quartile $-1.5 \times$ IQR. More extreme values are shown as individual points. Note the break on the y-axis. Because of the skewed distribution, the data were log-transformed before statistical analysis. The difference between the two groups is not significant.

whose tumor size did not change ($0.4 \pm 0.1\%$ vs. $0.8 \pm 0.3\%$, respectively).

Discussion

Our study demonstrates that octreotide-treated patients have lower Ki-67 LI in the pituitary tumor than matched untreated patients, but no significant difference in the apoptotic index.

Besides the well known regulation of hormone secretion, somatostatin and its analogs exert an antiproliferative activity on various tumors and human cancer cell lines *in vivo* and *in vitro* (24–28). Upon activation of the somatostatin receptors, whose presence has been detected in endocrine and nonendocrine human cancer (27, 29), the antiproliferative effect of somatostatin has been ascribed to direct antagonism of epidermal growth factor action in pancreatic tumor cells (30) and to stimulation of a phosphotyrosine phosphatase activity that, in turn, inhibits tyrosine kinase activity in other cell types (31, 32).

In the GH₃ rat pituitary tumor cell line, a frequently used cell model of GH-secreting tumors, octreotide induces a cytostatic block by inhibiting the entry of quiescent cells into the pool of replicating cells (33). Using the same cell system, another study demonstrated that octreotide prevented the irreversible passage of cells into S phase of the cell cycle by inhibiting either the expression of the early response gene *c-fos* or DNA binding of the heterodimeric transcription factor complex (34). Despite this experimental background, the antiproliferative effect of somatostatin and its analogs on GH-secreting adenomas has not been thoroughly investigated to date. In an uncontrolled study, the Ki-67 LI of 16 octreotide-treated acromegalics was half the value in 36 previously untreated patients (18). However, it was not clear whether the 2 groups of patients were comparable in terms of age, tumor size, tumor invasiveness, and pretreatment GH value. Thapar and co-workers (19) studied a subgroup of 32 acromegalic patients included in a multicenter randomized controlled trial of presurgical octreotide therapy. Octreotide treatment for 4 months before surgery was associated with a mean Ki-67 LI that was 83% that in surgical controls, independently of the densely or sparsely granulated nature of the tumors (19). Unfortunately, no information was available regarding the other clinical and hormonal characteristics. Our study confirms and extends these results. Despite the diverse nature of the studies (Refs. 18 and 19 and the present study), it is reassuring that the estimate of the antiproliferative effect of octreotide was reasonably similar in all 3 series (50%, 83%, and 53%, respectively). We used very stringent matching criteria to ensure that the 2 groups of patients differed only in the variable of interest, namely the presence or absence of octreotide pretreatment. The similarity of other characteristics, such as history of dopaminergic therapy, maximum tumor diameter, gonadal status at the time of surgery, and basal GH and PRL levels, which were not used as matching criteria, confirms that the 2 groups of patients were quite comparable. In the univariate analysis we found that tumor extension, defined as intrasellar vs. extrasellar extending tumors, and tumor invasiveness into the cavernous sinus affected the Ki-67 LI. Our data show that the

effect of octreotide pretreatment on Ki-67 LI occurred independently of either tumor extension or invasiveness. Together with the aforementioned effectiveness in both densely and sparsely granulated tumors (19), these results suggest that the antiproliferative effect of octreotide should occur in most GH-secreting adenomas. The possible influences of gonadal status on cell cycle characteristics are largely unknown. Our data, showing similar Ki-67 LI values in hypogonadal and eugonadal subjects and no interaction between gonadal status and octreotide treatment, suggest that the antiproliferative effect of the drug in acromegaly is virtually independent of patients' gonadal status. The principal potential bias of our study is the nonrandomized nature of octreotide treatment, which was usually decided by the referring endocrinologist, as well as the variability of the dose and the duration of medical treatment before surgery. The lack of a significant association between the Ki-67 LI and the total octreotide dose and duration of therapy seems to exclude an important confounding effect of the latter variables on our main results. The percentage of patients showing a marked reduction ($\geq 50\%$) of GH levels during octreotide therapy in our study (64%) is not much different from that in previous reports (7, 35, 36), suggesting that our case patients should not represent a particular subgroup of acromegalic patients. The proliferation index was significantly lower in the group of patients who reached an optimal level of GH suppression (basal GH, $< 2.5 \mu\text{g/liter}$) during octreotide treatment compared with patients with incomplete GH suppression, suggesting that the antiproliferative effect of octreotide in GH-secreting adenomas is more evident when GH secretion is almost completely suppressed. However, the relationship between the antiproliferative and antisecretory activities of octreotide is likely to be much more complex, as suggested by the lack of a significant difference in Ki-67 LI when patients were classified according to the criterion of a 50% reduction of GH levels. The time interval from the pretreatment MRI scan and that during octreotide therapy varied in our study population. Despite this possible caveat, we identified a reduction of the maximum tumor diameter in 5 of 25 evaluable patients (20%), a percentage slightly lower than that reported in other series (7–9). Interestingly, the Ki-67 LI was significantly lower in patients with shrinking adenoma compared with that in patients with no modification of tumor size. However, the low number of patients available for these analyses mandates caution in interpreting the relationship between the responsiveness to octreotide therapy and the antiproliferative effect of the drug.

The other possible mechanism by which octreotide might control the growth of GH-secreting adenomas is induction of apoptosis (16). It has been demonstrated in transfected CHO-K1 cells expressing the five known somatostatin receptors that octreotide in nanomolar concentrations can induce apoptosis by up-regulating p53 expression (37). However, only the somatostatin receptor subtype 3 seemed involved in signaling apoptosis (37). In another experimental model employing the murine ACTH-secreting adenoma cell line AtT-20, octreotide inhibited cell growth by inducing apoptosis during the G₂ phase of the mitotic cycle (38). The increase in the apoptotic index was about 5-fold after an incubation period of 24 h with the drug (38). Interestingly,

similar results were found in MCF-7 breast cancer cells treated in an analogous manner (39). High doses of lanreotide, another long-acting somatostatin analog, induced apoptosis in neuroendocrine gastrointestinal tumors, especially in those patients who showed a good clinical response to the drug (40). However, few data are available on the direct assessment of apoptosis in GH-secreting adenomas (20, 21, 41, 42). Our results, showing no significant difference in apoptotic index among octreotide-treated and untreated patients, are in keeping with data from other published reports (20, 21). In particular, Saitoh and co-workers (20) observed that the apoptotic index in 11 patients treated with 0.3 mg octreotide, sc, for 2–5 wk before surgery ($0.40 \pm 0.60\%$) was similar to that in 11 untreated patients ($0.81 \pm 0.79\%$). The absence of consistent morphological features among octreotide-treated tumors (10, 14) further supports the conclusion that the induction of apoptosis is not the principal mechanism by which the drug exerts its antiproliferative activity in GH-secreting adenomas. We did not find any significant association between the apoptotic index and several clinical characteristics of the patients. However, invasive tumors, in addition to a higher Ki-67 LI (see above), showed a higher apoptotic index than noninvasive tumors. It seems likely that the high apoptotic index of invasive adenomas is secondary to the increased proliferative activity of such tumors. This hypothesis is supported by the weak, but significant, correlation between apoptotic index and Ki-67 LI. Interestingly, a similar relationship between Ki-67 LI and apoptotic index was detected in a group of ACTH-secreting micro- and macroadenomas (23) and in malignant neoplasms at large (43, 44). It has been hypothesized that an increase in the proliferation activity within a cell population leads to exhaustion of factors that promote cell multiplication and inhibit apoptosis. Moreover, comparing normal and hyperplastic pituitaries, pituitary adenomas, and carcinomas, Kulig and co-workers (21) also detected a positive correlation between proliferative and apoptotic indexes, which seems to be a general rule in hormone-dependent epithelial tumors (45). GH and tumor responsiveness to octreotide, total dose, and duration of drug therapy did not affect the apoptotic index. However, we need caution in interpreting this result, as some of the associations were near the significance level. Because, as noted previously, different subtypes of somatostatin receptors may be involved in the regulation of the cell response, it is possible that somatostatin analogs with another binding profile, *i.e.* a higher affinity for somatostatin receptor subtype 3, will be shown to induce apoptosis in GH-secreting adenomas.

In conclusion, our study demonstrates that chronic octreotide administration has an antiproliferative effect on GH-secreting adenomas that is mainly mediated by a lower percentage of replicating cells, rather than stimulation of cell loss by apoptosis. From a clinical perspective, as treatment with somatostatin analogs needs to be lifelong to maintain adequate control of GH secretion, the antiproliferative effect of octreotide on GH-secreting adenomas, demonstrated in this and previous studies (18, 19), should reassure about the long-term control of tumor growth in treated patients.

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References

- Patel YC, Greenwood MT, Panetta R, Demchysyn L, Niznik H, Srikant CB 1995 The somatostatin receptor family. *Life Sci* 57:1249–1265
- Reisine T, Bell GI 1995 Molecular biology of somatostatin receptors. *Endocr Rev* 16:427–442
- Patel YC 1997 Molecular pharmacology of somatostatin receptor subtypes. *J Endocrinol Invest* 20:348–367
- Patel YC, Srikant CB 1994 Subtype selectivity of peptide analogs for all five cloned human somatostatin receptors (hsstr 1–5). *Endocrinology* 135:2814–2817
- Lamberts SWJ, Uitterlinden P, Verschoor L, van Dongen KJ, Del Pozo E 1985 Long-term treatment of acromegaly with the somatostatin analogue SMS 201–995. *N Engl J Med* 313:1576–1580
- Melmed S, Ho K, Klibanski A, Reichlin S, Thorner M 1995 Clinical Review 75. Recent advances in pathogenesis, diagnosis, and management of acromegaly. *J Clin Endocrinol Metab* 80:3395–3402
- Vance ML, Harris AG 1991 Long-term treatment of 189 acromegalic patients with the somatostatin analog octreotide: results of the international multicenter acromegaly study group. *Arch Intern Med* 151:1573–1578
- Sassolas G, Harris AG, James-Deidier A, et al. 1990 Long term effect of incremental doses of the somatostatin analog SMS 201–995 in 58 acromegalic patients. *J Clin Endocrinol Metab* 71:391–397
- Ezzat S, Snyder PJ, Young WF, et al. 1992 Octreotide treatment of acromegaly: a randomized, multicenter study. *Ann Intern Med* 117:711–718
- Barkan AL, Lloyd RV, Chandler WF, et al. 1988 Preoperative treatment of acromegaly with long-acting somatostatin analog SMS 201–995: shrinkage of invasive pituitary macroadenomas and improved surgical remission rate. *J Clin Endocrinol Metab* 67:1040–1048
- Asa SL, Felix I, Kovacs K, Ramyar L 1990 Effects of somatostatin on somatotroph adenomas of the human pituitary: an *in vitro* functional and morphological study. *Endocr Pathol* 1:236–244
- Beckers A, Kovacs K, Horvath E, Abs R, Reznik M, Stevenaert A 1991 Effect of treatment with octreotide on the morphology of growth hormone-secreting pituitary adenomas: study of 24 cases. *Endocr Pathol* 2:123–131
- Sautner D, Saeger W, Tallen G, Lüdecke DK, Rehpenning W 1993 Effects of octreotide on morphology of pituitary adenomas in acromegaly. *Pathol Res Pract* 189:1044–1051
- Ezzat S, Horvath E, Harris AG, Kovacs K 1994 Morphological effects of octreotide on growth hormone-producing pituitary adenomas. *J Clin Endocrinol Metab* 79:113–118
- Reubi JC, Landolt AM 1989 The growth hormone responses to octreotide in acromegaly correlate with adenoma somatostatin receptor status. *J Clin Endocrinol Metab* 68:844–850
- Landolt AM 1996 Growth of pituitary adenomas, malignant adenomas. In: Landolt AM, Vance ML, Reilly PL, eds. *Pituitary adenomas*. Edinburgh: Churchill Livingstone; 333–351
- Losa M, Franzin A, Mortini P, Terreni MR, Mangili F, Giovannelli M 1998 Usefulness of markers of cell proliferation in the management of pituitary adenomas. *Clin Sci* 95:119–125
- Landolt AM, Shibata T 1991 Growth, cell proliferation, and prognosis of pituitary adenomas. In: Faglia G, Beck-Peccoz P, Ambrosi B, Travaglini P, Spada A, eds. *Pituitary adenomas. New trends in basic and clinical research*. Amsterdam: Excerpta Medica; 169–178
- Thapar K, Kovacs K, Stefaneanu L, et al. 1997 Antiproliferative effect of the somatostatin analogue octreotide on growth hormone-producing pituitary tumors: results of a multicenter randomized trial. *Mayo Clin Proc* 72:893–900
- Saitoh Y, Arita N, Ohnishi T, Ekramullah S, Takemura K, Hayakawa T 1997 Absence of apoptosis in somatotropinomas treated with octreotide. *Acta Neurochir* 139:851–856
- Kulig E, Jin L, Qian X, et al. 1999 Apoptosis in nontumorous and neoplastic

- human pituitaries. Expression of the Bcl-2 family of proteins. *Am J Pathol* 154:767–774
22. **Cattoretti G, Becker MHG, Key G, et al.** 1992 Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB-3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* 168:357–363
 23. **Losa M, Barzaghi R, Mortini P, et al.** 2000 Determination of the proliferation and apoptotic index in adrenocorticotropin-secreting pituitary tumors: comparison between micro- and macroadenomas. *Am J Pathol* 156:245–251
 24. **Reubi JC** 1985 A somatostatin analog inhibits chondrosarcoma and insulinoma tumour growth. *Acta Endocrinol (Copenh)* 109:108–114
 25. **Prévost G, Foehrl E, Thomas F, et al.** 1991 Growth of human breast cancer cell lines is inhibited by the somatostatin analog BIM 23014. *Endocrinology* 29:323–329
 26. **Dy DY, Whitehead RH, Morris DL** 1992 SMS 201–995 inhibits *in vitro* and *in vivo* growth of human colon cancer. *Cancer Res* 52:917–923
 27. **Schally AV** 1998 Oncological applications of somatostatin analogs. *Cancer Res* 48:6877–6885
 28. **Pollak MN, Schally AV** 1998 Mechanism of antineoplastic action of somatostatin analogs. *Proc Soc Exp Biol Med* 217:143–152
 29. **Reubi JC, Laissue J, Krenning E, Lamberts SW** 1992 Somatostatin receptors in human cancer: incidence, characteristics, functional correlates and clinical implications. *J Steroid Biochem Mol Biol* 43:27–35
 30. **Lee MT, Liebow C, Kamer AR, Schally AV** 1991 Effects of epidermal growth factor and analogues of luteinizing hormone-releasing hormone and somatostatin on phosphorylation and dephosphorylation of tyrosine residues of specific protein substrates in various tumors. *Proc Natl Acad Sci USA* 88:1656–1660
 31. **Buscail L, Delesque N, Estève J-P, et al.** 1994 Stimulation of tyrosine phosphatase and inhibition of cell proliferation by somatostatin analogues: mediation by human somatostatin receptor subtypes SSTR1 and SSTR2. *Proc Natl Acad Sci USA* 91:2315–2319
 32. **Florio T, Scorizello A, Fattore M, et al.** 1996 Somatostatin inhibits PC C13 thyroid cell proliferation through the modulation of phosphotyrosine phosphatase activity. *J Biol Chem* 271:6129–6136
 33. **Cheung NW, Boyages SC** 1995 Somatostatin-14 and its analog octreotide exert a cytostatic effect on GH₃ rat pituitary tumor cell proliferation via a transient G0/G1 cell cycle block. *Endocrinology* 136:4174–4181
 34. **Todisco A, Campbell V, Dickinson CJ, Del Valle J, Yamada T** 1994 Molecular basis for somatostatin action: inhibition of c-fos expression and AP-1 binding. *Am J Physiol* 267:G245–G253
 35. **Fredstorp L, Harris A, Haas G, Werner S** 1990 Short term treatment of acromegaly with the somatostatin analog octreotide: the first double-blind randomized placebo-controlled study on its effects. *J Clin Endocrinol Metab* 71:1189–1194
 36. **Sassolas G, Fossati P, Chanson P, et al.** 1989 Experience of a six-month treatment with sandostatin at incremental doses in acromegaly. *Horm Res* 31:51–54
 37. **Sharma K, Patel YC, Srikant C** 1996 Subtype specific induction of wild type p53 and apoptosis, but not cell cycle arrest, by human somatostatin receptor 3. *Mol Endocrinol* 10:1688–1696
 38. **Srikant CB** 1995 Cell cycle dependent induction of apoptosis by somatostatin analog SMS 201–995 in AtT-20 mouse pituitary cells. *Biochem Biophys Res Commun* 209:400–406
 39. **Pagliacci MC, Tognellini R, Grignani F, Nicoletti L** 1991 Inhibition of human breast cancer cell (MCF-7) growth *in vitro* by the somatostatin analog SMS 201–995: effects on cell cycle parameters and apoptotic cell death. *Endocrinology* 129:2555–2562
 40. **Eriksson B, Renstrup J, Imam H, Öberg K** 1997 High-dose treatment with lanreotide of patients with advanced neuroendocrine gastrointestinal tumors: clinical and biological effects. *Ann Oncol* 8:1041–1044
 41. **Kontogeorgos G, Sambaziotis D, Piaditis G, Karameris A** 1997 Apoptosis in human pituitary adenomas: a morphologic and *in situ* end-labeling study. *Mod Pathol* 10:921–926
 42. **Green VL, White MC, Hipkin LJ, Jeffreys RV, Foy PM, Atkin SL** 1997 Apoptosis and p53 suppressor gene protein expression in human anterior pituitary adenomas. *Eur J Endocrinol* 136:382–387
 43. **Wyllie AH** 1985 The biology of cell death in tumours. *Anticancer Res* 5:131–136
 44. **Nakagawa S, Shiraishi T, Kihara S, Tabuchi K** 1995 Detection of DNA strand breaks associated with apoptosis in human brain tumours. *Virchows Arch* 427:175–179
 45. **Soini Y, Paakko P, Lehto V-P** 1998 Histopathological evaluation of apoptosis in cancer. *Am J Pathol* 153:1041–1053