Somatostatin Receptor sst2 Decreases Cell Viability and Hormonal Hypersecretion and Reverses Octreotide Resistance of Human Pituitary Adenomas

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Abstract
In human somatotroph adenomas, growth hormone (GH) hypersecretion can be inhibited by somatostatin analogues such as octreotide. Unfortunately, serum GH levels reach normal values in only 60% of treated patients. The decreased sensitivity to octreotide is strongly related to a lower expression of somatostatin receptor sst2. In this present study, the sst2 gene was transferred by an adenoviral vector (Ad-sst2) in human somatotroph (n = 7) and lactotroph (n = 2) adenomas in vitro. Sst2 mRNA levels and sst2 immunostaining dramatically increased after infection. Ten days after infection at 20 multiplicity of infection (MOI), sst2 gene transfer decreased cell viability from 19% to 90% by caspase-dependent apoptosis. At low viral doses (5 MOI), Ad-sst2 decreased GH or prolactin (PRL) basal secretion and mRNA expression. Somatotroph tumors were classified in three groups according to their octreotide sensitivity. Four days after infection by 5 MOI Ad-sst2, the maximal GH suppression by octreotide increased from 31% to 57% in the octreotide partially resistant group and from 0% to 27% in the resistant ones. In the octreotide-sensitive group, EC50 values significantly decreased from 1.3 \times 10^{-11} to 6.6 \times 10^{-13} mol/L without improving maximal GH suppression. Finally, lactotroph tumors, nonresponding to octreotide in basal conditions, became octreotide sensitive with a maximal PRL suppression of 43% at 10^{-8} mol/L. Therefore, sst2 reexpression is able to improve octreotide sensitivity. Sst2 gene transfer may open new therapeutic strategies in treatment combined with somatostatin analogues. [Cancer Res 2008;68(24):10163–70]

Introduction
Peptide-targeted therapy is a hot topic in Endocrine Oncology, with strong effect for both diagnostic and therapeutic strategies. The discovery of somatostatin analogues such as octreotide and lanreotide dramatically modified the treatment of endocrine tumors, in particular, pituitary and gastroenteropancreatic (GEP) tumors, through the control of symptoms due to excess hormone secretion and through the reduction of tumoral volume.

Somatostatin acts by binding five subtypes of G protein–coupled receptors (GPCR), sst 1, 2, 3, 4, and 5 that are widely distributed throughout many endocrine and nonendocrine tissues. Somatostatin analogues have a good sst2 affinity, 5- to 10-fold lower sst5 affinity, 50-fold lower sst3 affinity, but their affinities for sst1 and sst4 are not significant (1). The success of in vivo peptide-targeted therapy is highly dependent on the presence and on the localization in the tumor of a sufficient amount of the appropriate receptor. For somatotroph adenomas, we showed that the level of sst2 expression is correlated with the agonist sensitivity. These growth hormone (GH)-secreting tumors represent ~15% of pituitary adenomas (2). Often considered as benign, they can induce serious neurologic and metabolic complications. Serum GH and insulin-like growth factor 1 levels under octreotide reach normal values in only 60% of treated patients (3). Although a strong correlation exists between the level of sst2 mRNA expression and the percentage of GH inhibition under octreotide or native somatostatin (4, 5), no relationship has been observed between sst5 mRNA level and somatostatin sensitivity (5). In fact, the sst2 receptor is the main receptor mediating somatostatin-induced inhibition of GH secretion (6, 7).

Lactotroph and somatotroph cells derive from a common progenitor (8), and the majority of human somatotroph adenomas are somatolactotroph. Lactotroph adenomas are the most frequent pituitary tumors. The main pharmacologic treatment of these prolactin (PRL)-secreting adenomas is dopamine analogues. However, treatment is ineffective in 10% to 15% of treated patients, even with the most potent dopamine analogue, cabergoline (9, 10). Somatostatin receptors sst5, sst1, and sst2 are expressed in these tumors (11, 12). Besides the crucial role of sst2 in the response to somatostatin analogue in pituitary and GEP tumors, this receptor is a potential tumor suppressor gene for nonendocrine pancreatic adenocarcinoma (13–17). In vitro cell growth and in vivo tumorigenecity were strongly impaired in sst2-negative pancreatic cancer–derived cell lines genetically modified to express sst2 (13, 15–19). In this model, sst2 reexpression resulted in local production of somatostatin. Such an autocrine loop was thought to account for the antitumorigenic effect of sst2 (20). Therefore, besides pharmacologic approaches, gene therapy using the sst2 gene has been proposed as a potent antitumoral strategy (13).
Our aim was to introduce the sst2 gene in human somatotroph and lactotroph tumors by an adenoviral vector to control cell proliferation and secretion. Sst2 gene transfer was able to decrease cell viability by caspase-dependent apoptosis. At low viral doses, this transgene reduced GH and PRL basal oversecretion and rescued octreotide sensitivity.

Materials and Methods

Pituitary adenomas. The present study was approved by the ethics committee of the University and was undertaken after informed consent obtained from each patient and all participants. The study was carried out in pituitary macroadenomas, 7 somatotroph (A1-A7), and 2 lactotroph (P1, P2) tumors (Table 1), selected on the basis of clinical hormonal status and immunocytochemical data.

Cell culture. Fragments of human pituitary adenomas were dissociated mechanically and enzymatically (21). To eliminate fibroblast, anti-fibroblast MicroBeads (Anti-Fibroblast MicroBeads human; Miltenyi Biotec) were used according to the manufacturer’s protocol. Adenoma cells were plated on 24-, 12-, or 4-well dishes (according to the experiment) coated with extracellular matrix (ECM) of bovine corneal epithelial cells (21). Cells were cultured in DMEM, depleted in L-Valin (L-Valin was replaced by D-Valin to block fibroblast proliferation), and supplemented with high glucose (100 U/mL), and glutamine (100 U/mL). Cells were maintained at 37°C in an atmosphere containing 7% CO2.

Cell infection. To introduce the sst2 gene, an adenoviral vector was used because the proliferative rate of human pituitary cells is too weak in vitro (22) to use classic chemical transfection techniques.

After 24 h of culture, cells were infected with adenoviral vector containing either eGFP gene alone (Ad-eGFP) or mouse sst2 gene (Ad-sst2). Ad-sst2 vector also independently expressed eGFP. These transgenes were placed under the control of the human cytomegalovirus promoter. These vectors were previously described (17). Viral infections were done using various multiplicities of infection (MOI; from 5–20 MOI) in a final volume of 300 µL of complete medium. In noninfected wells, 300 µL of complete medium alone was added. Two and half hours after infection, the viral suspension was replaced by 1 mL of complete culture medium in infected as well as in noninfected wells. In all experiments, Ad-sst2–infected cells were compared with Ad-eGFP–infected cells and with noninfected cells.

Flow cytometry analysis. To determine the transduction efficiency of the adenoviral vectors, fluorescence-activity cell sorting (FACS) analysis was done. Five days after infection by Ad-eGFP or Ad-sst2, 1 × 10^5 cells were trypsinized and collected in tube containing 800 µL of complete culture medium with propidium iodide (PI; 0.5 µg/mL; BD Pharmingen) and run on FACS sort (Becton Dickinson). Data were analyzed with the Cell Quest program (Becton Dickinson). Ten thousand events were acquired for each analysis and assayed in duplicate.

RNA extraction and real-time PCR. Sst2, GH, PRL, and somatostatin mRNA expression were assessed in duplicate using real-time PCR. In brief, 2.5 × 10^5 cells per well were plated in 12-well dishes. Four days after infection, total mRNAs were extracted using the RNeasy Micro kit (Qiagen) from cells. Total RNA was reverse-transcribed into complementary DNA (cDNA) using 200 IU Superscript II Reverse Transcriptase (Invitrogen) primed with 300 ng/mL of random hexamer primer, according to the manufacturer’s protocol. The 5’ exonuclease (Taq man) assay was used to quantify sst2 mRNA as previously described (23) and to quantify PRL mRNA (Hs 00168730-m1; PE Applied Biosystems). Primers and probes in sst2 real-time PCR were designed to recognize both hsst2 and msst2. The amplifications of msst2 and hist2 plasmid γ displayed the same efficiency (data not shown). The SYBR Green assay purchased from Qiagen was used to quantify GH (Hs-GH-Gr; QuantTect Primer Assay) and somatostatin (Hs-SST 5G; QuantTect Primer assay). Forty cycles of two-step PCR-annealing extension were performed on an ABI Prism 7700 sequence detection apparatus (PE; Applied Biosystems Paris). The mRNA levels were normalized to the β-glucuronidase (βGus) mRNA levels (23). To produce standard curves, cDNA constructs were produced for each variable, verified by sequencing (Beckman Coulter Ceq 8000), and linearized. The results were expressed as copy of gene/copy of βGus.

Immunocytochemistry. After infection, the expression and localization of sst2 were assessed by immunocytochemistry. Adenoma cells were cultured on ECM-coated 14-mm cover glass. Then, cells were infected with Ad-eGFP or Ad-sst2 at 5 MOI or were not infected. Forty eight hours later, cells were fixed, counterstained with Hoechst 33342 (1 mg/mL) for 5 min at room temperature, and incubated overnight at 4°C with an antisense against sst2A (SS-800; Gransch Lab, Germany; ref. 24), diluted at 1:1,000 in PBS supplemented with 1% bovine serum albumin (Sigma). The immunostaining was visualized using Alexa 594–conjugated goat anti-rabbit IgG (Molecular Probes, Invitrogen) diluted at 1:500 in PBS containing 10% normal goat serum. A peptide fragment of somatostatin receptor (S8–801, Gransch Lab, Germany) was used to test anti-sst2 specificity. This peptide was incubated overnight (×100 or ×1,000) with anti-sst2, and then cells were incubated with this mix. Confocal image acquisition was performed on a Leica TCS SP2 laser scanning microscope, and image editing was performed using Adobe Photoshop. To quantify sst2

<table>
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<th>Case no</th>
<th>Age (Yr)</th>
<th>Sex</th>
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<th>PRL (µg/L)</th>
<th>IGF-I (µg/L)</th>
<th>GH (µg/L)</th>
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<td>F</td>
<td>32</td>
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<td>37</td>
<td>60</td>
<td>993</td>
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<td>25000</td>
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<td>ND</td>
<td>3150</td>
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</table>

NOTE: M, male; F, Female. ND, not determined.

Table 1. Clinical characterization and hormonal secretion in vivo and in vitro in patients bearing somatotroph (A) and lactotroph adenomas (P)
has been shown to be selective for caspases 3 and 7. Each experimental condition was assayed in triplicate wells containing 5 × 10^5 adenoma cells. After 24 h of culture, cells were infected with various MOI of Ad-eGFP or Ad-sst2 or were not infected. The results were expressed as mean percentage of cell viability versus noninfected cells. A direct cell count was also performed on three tumors by a Coulter apparatus (Beckman-Coulter).

Hormonal release. For hormonal (GH and PRL) release assessment, the medium of the 5 × 10^5 cells noninfected and infected by Ad-eGFP or Ad-sst2 at 20 MOI was collected at 3, 7, and 10 d after infection and stored frozen for hormonal measurement. GH and PRL measurements were performed using commercial immunoradiometric kits (Immunootech Marseille Beckman Coulter). For somatostatin release assessment, the medium of 2.5 × 10^5 cells was collected 4 d after infection, and measured after ethanol extraction using the RIA kit with a sensitivity at 5 pmol/L (EUPIA-somatostatin; EURO-DIAGNOSTICA AB).

Cell cycle analysis. For cell cycle distribution analysis, noninfected cells and cells infected with Ad-eGFP or Ad-sst2 at 20 MOI were harvested by trypsin treatment 10 d after infection and fixed in ethanol 95% for 30 min on ice. Then, cells were treated with RNaseA (1 mg/ml) for 30 min at room temperature. DNA was stained with PI (25 μg/ml), protected from light before FACS analysis. DNA cell distribution histograms were analyzed using the CellQuest Pro software program (Becton Dickinson). Each experimental condition was assayed in triplicate.

Detection of caspase-dependent apoptosis. To determine the mechanism of cell death, DNA fragmentation was analyzed. Eighty thousand adenoma cells were plated on ECM-coated 14-mm cover glass. Cells were infected with Ad-eGFP or Ad-sst2 at 20 MOI. Noninfected cells were used as control. Six days later, cells were fixed 15 min with paraformaldehyde. DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) using ApopTag Red In Situ Apoptosis Detection Kit (Chemicon International, Inc.). Each experimental condition was assayed in quadruplicate. Apoptotic cells were then viewed and scored manually with a Leica/Leitz DMRB microscope using a PL fluorot 100 objective. The percentage was evaluated on the basis of ≥3,000 counted cells in 70 to 120 successive fields.

To evaluate the executioner caspase activities, 5 × 10^4 cells were infected by 20 MOI Ad-eGFP or 20 MOI Ad-sst2 or were not infected. After 6 d, caspase activity was measured using the Caspase-Glo 3/7 Assay (Promega Corporation) containing the substrate tetrapeptide sequence DEVD, which has shown to be selective for caspases 3 and 7. Each experimental condition was assayed in triplicate wells. Caspase activity was normalized to the cell number determined by CellTiter-Glo assay.

Octreotide sensitivity. To assess the octreotide sensitivity, 2 × 10^5 cells per wells were plated in 24-well dishes. After 24 h of culture, cells were infected with Ad-eGFP or Ad-sst2 at 5 MOI or were not infected. After 3 d, the effect of various doses of octreotide (1 × 10^-12 to 1 × 10^-6 mol/L) were tested for their ability to inhibit GH or PRL release over an 8 h period, in DMEM containing 1% FCS. Moreover, for the 2 prolactinomas, the effect of a 1 × 10^-7 mol/L dose of cabergoline (provided by Pfizer, Pharmacia & Upjohn) was tested on the PRL release over an 18-h period. Each condition was tested in triplicate. Culture medium were then collected and stored frozen for GH and PRL measurements.

Statistical analysis. The results are presented as the mean ± SE. The statistical significance between two paired groups was determined by the Wilcoxon nonparametric test, and those between two unpaired groups by Mann-Whitney nonparametric test or by Student’s t test. To measure the strength of association between pairs of variables without specifying dependency, Spearman rank order correlations were run. Differences were taken to be statistically significant at a probability level of <0.05.

Results

Adenoviral transduction efficiency and sst2 transgene mRNA and protein expression. The transduction efficiency of the adenoviral vectors in human pituitary cells in vivo was evaluated by FACS analysis in four tumors (A1, A3, A7, and P2). Four days after infection, the mean percentage of eGFP-expressing cells was 61.1% ± 10% and 60.8% ± 9% after infection by 5 MOI Ad-eGFP and 5 MOI Ad-sst2, respectively, and increased up to 99% at 20 MOI. Moreover, 10 days after infection, eGFP expression was still present (data not shown). In noninfected cells, no eGFP expression was detected.

To follow up sst2 transgene expression, sst2 mRNA was quantified by real-time PCR in 5 MOI Ad-sst2–infected cells, compared with 5 MOI Ad-eGFP–infected cells and to noninfected cells (Table 2). Endogenous sst2 mRNA expression was found in all tumors (Table 2, noninfected) and was not significantly different from that found in Ad-eGFP–infected cells (mean sst2 mRNA levels, 159 ± 46 × 10^-2 and 163 ± 46 × 10^-2 copy/copy βGus, respectively; Table 2). Sst2 mRNA levels dramatically increased after infection by Ad-sst2 (mean sst2 mRNA level, 14,025 ± 4,558 × 10^-2 copy/copy βGus), in a highly variable manner dependent on each tumor (P < 0.004; Table 2).

The expression of sst2 protein was assessed by immunocytochemical analysis in three tumors (A3, A5, and P1). Forty eight hours after infection by Ad-sst2 at 5 MOI, the immunofluorescent staining was clearly more intense in Ad-sst2–infected cells (Supplementary Data S1 for A5; Fig. 1B for A3 and D for P1) than in noninfected cells (Supplementary Data S1 for A5; Fig. 1A for A3 and C for P1) or in cells infected by Ad-eGFP (data not shown). In tumor A3, the quantification showed a 13-fold in sst2 immunostaining increase after Ad-sst2 infection. The signal disappeared with sst2 peptide fragment (data not shown). The weak staining observed in noninfected cells seemed mostly in the perinuclear golgi area; in contrast, in Ad-sst2–infected cells, the strong staining was cytoplasmic and at the membrane. These data clearly showed the transduction efficiency and the transgene sst2 expression after adenoviral infection.

sst2 effect on cell viability. To determine whether sst2 transgene affected cell viability, an increasing viral dose (5–20 MOI) of Ad-eGFP or Ad-sst2 was applied on one somatotroph adenoma (A5). Ten days after infection, cell number was assayed using indirect estimates of the number of viable cells based on a luminescent cell viability assay. Cell number was lower for Ad-sst2–infected cells than for Ad-eGFP–infected cells and noninfected cells (Table 2).

In vitro the adenoviral vectors in human pituitary cells

http://rsb.info.nih.gov/ij/
significant effect was observed after Ad-eGFP transduction. Direct
cell counts on three adenomas (A3, A6, and P2) in the same expe-
rimental conditions of infection yielded similar results (Supple-
mentary Data S2). These data underlined the clearly negative effect
of Ad-sst2 infection on cell viability of pituitary adenoma in vitro.

Sst2 proapoptotic effect involving executioner caspase.
To determine the mechanism by which sst2 transgene induced a
negative effect on cell viability, somatotroph cells transduced by

Table 2. Sst2 mRNA levels in pituitary adenoma cells
in vitro

<table>
<thead>
<tr>
<th>Case no</th>
<th>non infected</th>
<th>Ad-eGFP</th>
<th>Ad-sst2</th>
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<tr>
<td>A1</td>
<td>420</td>
<td>410</td>
<td>12 836</td>
</tr>
<tr>
<td>A2</td>
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<tr>
<td>P2</td>
<td>1.2</td>
<td>1.7</td>
<td>23 813</td>
</tr>
</tbody>
</table>

NOTE: The quantification was performed by real-time PCR 4 d after
infection with Ad-eGFP or Ad-sst2 at MOI 5 and in non infected cells.
Results were expressed as 10^{-2} copy/copy βGus.

20 MOI Ad-eGFP or 20 MOI Ad-sst2 and noninfected cells were
examined using flow cytometry methods after undergoing DNA
staining with PI (Fig. 2C). Ten days after infection, the percentages
of cells in G1-S and in G2-M phase were slightly affected by Ad-sst2
transduction, whereas those in subdiploid sub-G1 phase (repre-
senting cell death) markedly increased (Fig. 2C). The percentage
of cells in each phase of cell cycle was highly similar for noninfected
cells and Ad-eGFP–infected cells. To confirm the apoptosis induced
by sst2 overexpression, DNA fragmentation was investigated using
TUNEL assay 6 days after infection at 20 MOI. A larger number of
apoptotic events per field occurred in cells transduced by Ad-sst2
(mean of apoptotic events, 4.02 ± 0.35 versus 0.86 ± 0.16 in
Ad-eGFP–infected cells; P < 0.001; Fig. 2D, left). In noninfected
(data not shown), and in Ad-eGFP–infected cells, the number of
apoptotic events observed was similar.

Finally, the apoptotic effect of sst2 was investigated by
measuring the activity of executioner caspase, 6 days after Ad-
sst2 infection. In same experimental conditions as above, Ad-sst2
infection increased the basal caspase activity (noninfected cells)
5-fold, but no significant effect was observed after Ad-eGFP infec-
tion (Fig. 2D, right).

Sst2 effect on hormonal basal secretion and expression.
Four days after infection at 5 MOI (a viral dose without effect on
cell viability), Ad-sst2–infected cells presented a significant
decrease in GH or in PRL mRNA levels in three tested tumors
(A2, A3, and P1). The mean percentages of GH or PRL mRNAs
decrease versus noninfected cells were, respectively, 48.7 ± 21%
in somatotroph tumors and 36% ± 18% in the lactotroph one,
whereas no significant effect was observed in Ad-eGFP–infected

Figure 1. Confocal microscopy
sections (800X) of sst2 (red emission)
immunocytochemistry analysis in
noninfected cells (A and C), and in cells
infected (B and D) by 5 MOI Ad-sst2 from
one human somatotroph (A3; A and B)
and one human lactotroph (P1; C and D)
adenoma. Immunocytochemical analysis
was done 48 h after infection. The nuclei
were in blue due to Hoechst coloration.
Scale bars, 10 μm.
levels were 0.2 was not modified by sst2 infection (the mean somatostatin mRNA expression level of somatostatin was very low in the 9 tumors and real-time PCR in the cultured cells from all tumors. The basal and release.

produced an inhibitory effect on both secretion and expression of GH These data showed that sst2 transgene overexpression alone pro-

duced an inhibitory effect on both secretion and expression of GH and PRL.

The mean percentages of hormone inhibition versus noninfected cells were 40% F

and PRL. In each tumor, the dose-related hormonal suppression was similar

infection with a maximal hormonal suppression of 27% under octreotide treatment. A dose-response hormonal suppression curve was assessed in the 7 somatotroph and in the 2 lactotroph adenomas, 4 days after infection with Ad-eGFP or Ad-sst2 at 20 MOI. For TUNEL assay, the results are expressed as the number of positive events per field. For caspase activities, the results were normalized to cell number (determined by celltiter-Glo assay) and expressed in percentage of caspase activity versus caspase activity of noninfected cells. Each experimental condition was assayed in triplicate. Columns, mean of 3 tumors (*, P < 0.001); bars, SE.

Figure 2. Ad-sst2 transduction decreased cell viability of human pituitary tumoral cells by an apoptotic-caspase dependent pathway. A and B, cell viability was monitored by luminescent cell viability assay 10 d after infection. Results are expressed as percentage of cell viability versus noninfected cells. A, dose response curve of Ad-sst2 and Ad-eGFP infection in one human somatotroph adenoma (A5; *, P < 0.02 compared with Ad-eGFP–infected cells). B, cell viability after infection by Ad-sst2 or by Ad-eGFP at 20 MOI in the 7 somatotroph and the 2 lactotroph adenomas (P = 0.008 compared with Ad-eGFP–infected cells). C, cell cycle distribution analysis 10 d after infection by Ad-eGFP or Ad-sst2 at 20 MOI and in noninfected cells. Cell cycle distribution was analyzed by flow cytometry. Results represent the mean of 3 somatotroph tumors and are expressed in percentage of cells present in each phase of cycle (*, P < 0.05 compared with noninfected cells or to Ad-eGFP–infected cells). D, left, DNA fragmentation analysis by TUNEL assay; right, caspase activities monitored by Caspase-Glo assay, 6 d after infection with Ad-eGFP or Ad-sst2 at 20 MOI. For TUNEL assay, the results are expressed as the number of positive events per field. For caspase activities, the results were normalized to cell number (determined by celltiter-Glo assay) and expressed in percentage of caspase activity versus caspase activity of noninfected cells. Each experimental condition was assayed in triplicate. Columns, mean of 3 tumors (*, P < 0.001); bars, SE.

cells (10.6% ± 9% and 9.5 ± 34%, respectively). The decrease in hormonal mRNA expression was associated with an inhibition in hormonal release as presented in Fig. 3 for the three tumors. The mean percentages of hormone inhibition versus noninfected cells were 40% ± 23% for GH and 52% ± 10% for PRL at 10 d after Ad-sst2 infection. GH and PRL release in the medium of Ad-eGFP–infected cells and in the medium of noninfected cells was similar. These data showed that sst2 transgene overexpression alone produced an inhibitory effect on both secretion and expression of GH and PRL.

Sst2 effect seemed independent of somatostatin expression and release. Somatostatin mRNA expression was measured by real-time PCR in the cultured cells from all tumors. The basal expression level of somatostatin was very low in the 9 tumors and was not modified by sst2 infection (the mean somatostatin mRNA levels were 0.2 ± 0.2 × 10^{-2}, 0.7 ± 0.8 × 10^{-2}, and 0.3 ± 0.3 × 10^{-2} copy/copy βGus for noninfected cells, Ad-eGFP–infected cells, and Ad-sst2–infected cells, respectively). Moreover, the basal release of endogenous somatostatin was assayed in the culture medium of cells infected by 5 or 20 MOI of Ad-sst2 or Ad-eGFP and of noninfected cells. The somatostatin levels were undetectable (below 5 pmol/L) in all conditions.

Sst2 effect on octreotide sensitivity. According to the in vitro results of GH release inhibition in noninfected cells under 10^{-10} mol/L octreotide, somatotroph adenomas were classified in three groups: sensitive (A1 and A2), partially resistant (A3, A4), and resistant tumors (A5, A6, and A7) with a mean percentage of GH inhibition by octreotide of 32.5% ± 3.5%, 10% ± 6%, and 0.6% ± 1.5%, respectively. The effect of Ad-sst2 infection was then analyzed on the hormonal secretion under octreotide treatment. A dose-response hormonal suppression curve was assessed in the 7 somatotroph and in the 2 lactotroph tumors, 4 days after infection at 5 MOI (by Ad-sst2 or Ad-eGFP) and in noninfected cells. In each tumor, the dose-related hormonal suppression was similar for Ad-eGFP–infected cells and noninfected cells. In the octreotide partially resistant group, the maximal GH suppression under octreotide increased significantly from 31% ± 9% for Ad-eGFP–infected cells to 57% ± 14% for Ad-sst2–infected cells (P < 0.05; Fig. 4B). Moreover, the dose-related curve of GH suppression was displaced on the left for Ad-sst2–infected cells versus Ad-eGFP–infected cells or noninfected cells (EC_{50} 6.7 × 10^{-10} versus 2.3 × 10^{-11} mol/L). The completely resistant somatotroph tumors became partially sensitive to octreotide after Ad-sst2 infection with a maximal hormonal suppression of 27% ± 7% at 10^{-10} mol/L (P < 0.05; Fig. 4C). In the octreotide sensitive somatotroph tumors, the maximal GH suppression was similar in the Ad-sst2, Ad-eGFP, and noninfected cells. However, the octreotide showed efficacy at lower concentrations, as shown by a lower EC_{50} (EC_{50} 6.6 × 10^{-13} mol/L for Ad-sst2–infected cells versus 1.3 × 10^{-11} mol/L for Ad-eGFP–infected cells and noninfected cells;
Finally, the lactotroph tumors, which did not respond to octreotide in basal conditions, became responsive to octreotide after Ad-sst2 infection with a maximal PRL suppression of 43.4% \( \pm \) 7%; this PRL suppression was close to that obtained with dopamine analogue (PRL suppression under 10 \( \times \) 10\(^{-9} \)) mol/L of cabergoline, 52% \( \pm \) 5%, Fig. 4D).

**Discussion**

In human somatotroph tumors, somatostatin-resistant tumors are the biggest and the most aggressive (25), suggesting that the loss of sst2 expression may confer these tumors a growth advantage. In this study, we showed that sst2 gene transfer alone induced a strong decrease in cell viability of human somatotroph and lactotroph adenomatous cells in vitro. It is well-known that somatostatin and its analogues may trigger antiproliferative signaling through cytostatic (growth arrest) and cytotoxic (apoptosis) effects in somatotroph cells (26–28). In our experimental conditions, the cell cycle analysis revealed that the main process in cell viability inhibition was cell death referring to the increase in sub-G1 population. Only a slight but not significant reduction of the proportion of cells present in G2-M fraction was observed under sst2 overexpression. The weakness of sst2 effect in G2-M phase probably results from the weak proliferative potential of human adenoma cells in vitro (22). The TUNEL analysis confirmed the DNA damage involving the executioner caspase activation. In other cellular models such as pancreatic cancer cells, several arguments favor the sst2 role as a tumor suppressor (29). In these cells, the apoptotic effect of sst2 involved both death ligand and mitochondrial pathways (30), both of which remain to be explored in our pituitary models.

In the pancreatic model, the tumor suppressor effects of sst2 are based on an sst2-activated autocrine loop; transfection of sst2 in cells lacking this receptor induces expression of its own ligand somatostatin, which in turn constitutively activates sst2 (13, 16). Moreover, disruption of this autocrine loop by RNA interference reversed sst2 antitumoral activity (20). However, in our experimental conditions, somatostatin immunoreactivity was not detected in the medium of human pituitary cell culture, although the sensitivity of the method was 5 pmol/L. According to previous works, somatostatin basal secretion can be achieved at a level 1,000-fold lower than the GH secretion basal level (31), which, in our culture conditions, could account for 60 nmol/L. In fact, only a very weak
expression of somatostatin mRNA was detected by Q-PCR in human pituitary cells in our experimental conditions. This expression was not modified at all by sst2 transduction. In agreement with our results, no somatostatin immunoreactivity was detected in the hamster pancreatic tumors bearing sst2, although the antitumorigenic effect was clear (15). Our data suggest a ligand-independent sst2 effect, i.e., a receptor constitutive activation. Very recently, using a small inhibitory RNA (siRNA) approach, Ben-Shlomo and colleagues (32) showed sst2, sst3, and sst5 had a ligand-independent receptor-constitutive action. Under selective sst2, sst3, or sst5 siRNAs, the basal levels of cyclic AMP, extracellular signal-regulated kinase 1/2 phosphorylation, and adrenocorticotrophic hormone secretion increased in the AtT-20 corticotroph cell line. In our study, the dramatic increase in sst2 mRNA levels after Ad-sst2 infection may favor receptor homodimerization. Independently or dependently relative to homodimerization, sst2 overexpression may trigger signal transduction pathways without external signal. The concept of constitutively active GPCR is now firmly rooted in receptor pharmacology, as revealed by the inverse agonists discovered for many receptors (33).

Independently of cell death, at low viral doses, we showed that sst2 transgene induced a decline of hormonal basal secretion after infection. This effect was associated with a decrease in GH and PRL expression. The inhibitory effect of sst2 gene transfer was obtained by using viral doses 2- to 4-fold lower than those used for cell death effects. This suggests different signal transduction pathways are involved for antihormonal and antitumoral effects. Moreover, note that Ad-sst2 was able to decrease cell viability even in somatotroph octreotide resistant tumors for which GH basal secretion was unchanged under octreotide (as A5, A6, and A7). Again, this suggests there is a partition between transduction pathways involved in cell death process and those activated during GH secretion inhibition by octreotide. In GH-secreting adenomas, somatostatin analogues produce their apoptotic action in a phosphatase-dependent manner (27), whereas the somatostatin inhibition of hormone secretion depends on the inhibition of adenylate cyclase and/or the regulation of ion channels (34).

For the first time, we show that the reinduction of sst2 expression is able to restore the octreotide response of partially resistant somatotroph tumor. The maximal inhibitory effect under octreotide increased significantly after Ad-sst2 infection, whereas the EC50 became 10-fold lower. Therefore, the in vitro dose response curves of these tumors became similar to those of tumors sensitive to octreotide in vivo (5, 25). These data point out the crucial role of sst2 in the capacity of the tumor to respond to octreotide. In the octreotide sensitive group, octreotide was efficient at lower concentrations after Ad-sst2 infection without increasing the maximal inhibition. In the octreotide fully resistant group, a partial restoration of octreotide sensitivity was observed; the pattern of response become close to that of partially sensitive tumors. The sst2 expression and cellular sst2 localization did not differ for partially resistant and resistant tumors (Supplementary Data S1). One may therefore hypothesize alterations occur in the transduction pathways downstream from the receptor. It has already been proposed that somatostatin postreceptor alterations occur in human somatotroph adenomas (4, 35). For octreotide resistant tumors, it has been shown that there is a coordinated decrease in expressions of several genes coding for proteins implicated in the processes of differentiation and secretion of somatotroph cells (4). Similarly, for human dopamine–resistant prolactinomas, we have previously showed expression is decreased for the messengers coding for Goe2 and Pit-1, proteins downstream from the D2 dopamine receptor in the transduction pathways (36, 37).

Prolactinoma tumors do not usually respond to octreotide (12). Induced sst2 expression allowed octreotide to be fully effective in this type of tumors. However, PRL suppression by octreotide did not exceed that by dopamine. The difference in the restoration of octreotide sensitivity between the two prolactinomas, and the three GH octreotide resistant tumors suggests the transduction

![Figure 4](https://www.aacrjournals.org)
pathways triggered by this peptide are present in prolactinomas but might be modified in GH octreotide–resistant tumors. In conclusion, sst2 overexpression induced cell death of human somatotroph and lactotroph tumoral cells. Our data suggest this receptor has an intrinsic activity. Thus, the loss of sst2 expression during pituitary tumorigenesis could play a role in the deregulation of cell growth and hormonal secretion not only by the suppression of somatostatin-induced inhibitory signaling but also by the loss of sst2 itself, which can thus be considered a suppressor gene in this study. Moreover, sst2 re-expression is able to rescue the octreotide sensitivity at low viral charge. It could be hazardous to translate these in vitro data to the in vivo conditions. Moreover the studies on pituitary gene therapy is still at an early stage. Because the adenosinergic vectors used in this study can elicit severe inflammatory responses, (38), concerns have been raised about the safety of the first generation of such adenosinergic vectors. Nevertheless, with a more appropriate vector, our data may position sst2 as a new candidate for gene therapy for pituitary tumors but also for a wide number of tumors. In new therapeutic strategies, a combined treatment included sst2 gene transfer with somatostatin analogues or with somatostatin analogues coupled to radioisotopes could be imagined.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

References


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Somatostatin Receptor sst2 Decreases Cell Viability and Hormonal Hypersecretion and Reverses Octreotide Resistance of Human Pituitary Adenomas

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