

Characterization of the functional and growth properties of long-term cell cultures established from a human somatostatinoma

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Abstract

In somatostatinoma, a rare malignant somatostatin (SST)-secreting neoplasia, tumour regression is rarely observed, implying the need for novel antiproliferative strategies. Here, we characterized a long-term culture (SST-secreting cancer (SS-C cells)) established from a human somatostatinoma. High concentrations of SST and chromogranin A were released by SS-C cells and SST release was stimulated by depolarizing stimuli and inhibited by the SST analogue, octreotide. SS-C cells expressed mRNA for SST receptor (SSTR) subtypes 1, 2 and 4, being also able to bind native SST. Moreover, SS-C cells were positively stained with an antibody to SSTR2. SS-C cells also expressed interferon- γ (IFN- γ) receptor mRNA and measurable telomerase activity. Our findings indicate that *in vitro* exposure of SS-C cells to native SST-28, to octreotide, to IFN- γ , or to 3'-azido-3'deoxythymidine (AZT), a telomerase inhibitor, results in inhibition of SS-C cell proliferation. Concomitant with growth inhibition, apoptosis was detected in SST-, octreotide-, IFN- γ - or AZT-treated SS-C cell cultures. Taken together our results characterized native SST, SST analogues, IFN- γ and a telomerase inhibitor as growth-inhibiting and proapoptotic stimuli in cultured human somatostatinoma cells. Based on these findings, the potential of SST analogues, IFN- γ and AZT, alone or in combination, should be further explored in the medical treatment of somatostatinoma.

Endocrine-Related Cancer (2006) 13 79–93

Introduction

Somatostatinoma is a rare malignant neuroendocrine tumour, accounting for fewer than 1% of all gut and pancreatic endocrine neoplasia (Jensen & Norton 1993), with preferential localization in the pancreas, followed by duodenum and jejunum (Konomi *et al.* 1990, Bordi *et al.* 1996). The lack of specificity of symptoms makes difficult an early and preoperative diagnosis of this tumour (Sassolas & Chayvialle 1995). Therefore, in more than 85% of the cases the diagnosis is established when liver and lymph node metastases

are displayed. *Ex vivo* and *in vivo* analyses of somatostatinoma tissues and cells showed the presence of somatostatin (SST) and interferon- γ receptors (IFN- γ -Rs), opening the possibility of treatment of metastatic disease with SST analogues and IFN- γ (Angeletti *et al.* 1998, Detjen *et al.* 2002).

The availability of *in vitro* models of somatostatinoma cells would certainly increase the possibility of treatment with potential pharmacological agents. The only model of a cultured human SST-secreting cell line obtained from a neuroendocrine pancreatic tumour, QGP-1 cells, was described more than a

decade ago (Iguchi *et al.* 1990). The QGP-1 cell line was characterized by a slow growth rate, by the production of SST, of carcinoembryonic antigen (CEA) and of small quantities of pancreatic polypeptide (PP) (Iguchi *et al.* 1990) and by the expression of functional IFN- γ -Rs (Detjen *et al.* 2002).

In this study we examined the functional and growth properties of long-term cell cultures of an SST-secreting cancer (SS-C cells) obtained from a primary human somatostatinoma. We also evaluated the response of SS-C cells to various physiological and pharmacological agents.

Materials and methods

Patient's history

Tissue samples were obtained from a female patient (aged 28 years) affected by malignant metabolic jejunal somatostatinoma after surgical removal of the tumour. The patient had neither a family history of endocrine pathology nor associated endocrinopathies. Diagnosis was made on the basis of clinical and biochemical characteristics of the patient in combination with (immuno)histochemistry of the primary tumour and of the liver metastatic tissues. Tests performed included RIA of hormones, computed tomography (CT), magnetic resonance imaging (MRI), octreotide scintigraphy, endoscopic ultrasonography, genetic mutational test for *MEN1* gene, and immunohistochemical staining of tumour tissue. The patient gave her informed consent for the use of tumour material for research purpose.

Materials

SST-28 was purchased from Calbiochem (Langentigen, Switzerland); recombinant human IFN- γ was purchased from Boehringer Mannheim (Mannheim, Germany); octreotide was supplied by Novartis (Basel, Switzerland); SST-28, octreotide and IFN- γ were dissolved in 0.01 M acetic acid containing 0.1% purified serum albumin and then stored at -80°C as 1 mM solutions. Fresh working solutions were prepared as new aliquots for each experiment. The Ultravision kit was from Lab Vision (Fremont, CA, USA); clone LKzH10 and anti-gastrin antibody from BioGenex (San Ramon, CA, USA); annexin V-FITC was obtained from Caltaq Laboratories (Burlingame, CA, USA); radioimmunological kits were: for somatostatin, cholecystokinin (CK) and substance P, from Euro-Diagnostica (Malmo, Sweden), for PP and gastric inhibitor peptide (GIP) from Phoenix Pharmaceutical (Belmont, CA, USA), for vasoactive intestinal

peptide (VIP) from Diasorin (Stillwater, MN, USA), for calcitonin gene-related peptide (CGRP), from Peninsula Laboratories Inc. (Belmont, CA, USA), for glucagon from Adaltis (Casalecchio di Reno, Italy), for gastrin from Incstar Co. (Stillwater, MN, USA), for chromogranin A (CgA) from Schering S.A. (Cedex, France), for calcitonin from Scantibodies Laboratory Inc. (Santee, CA, USA), for neuron-specific enolase (NSE) from CIS Bio International (Cedex France), and for C-peptide and insulin from TecnoGenetics (Cassina de'Pecchi, Italy). Polyclonal antibodies against somatostatin, glucagon, gastrin, and PP were obtained from DAKO (Carpinteria, CA, USA), while those for synaptophysin and NSE were from Ventana Medical Systems Inc. (Strasbourg, France). *TP53* gene mutation screening kit was purchased from Analytica (Padua, Italy). Clone AMV First-Strand CDNA synthesis kit was from Invitrogen Corporation (Carlsbad, CA, USA). RNAWIZ RNA Isolation Reagent was from Ambion, Inc. (Austin, TX, USA). The QUIAEXII kit was from Qiagen Inc. (Valencia, CA, USA) and puReTaq Ready-To-Go PCR beads from Amersham Biosciences Corp. (Piscataway, NJ, USA). Annexin V-FITC was from Caltaq Laboratories. The ligand, 3-[^{125}I]iodotyrosyl 25 -somatostatin-28 (Leu 8 ,D-Trp 22 ,Tyr 25), was obtained from Amersham (Milan, Italy) and was used at a final concentration of 0.1 nM. An antibody to SST receptor type 2 (SSTR2) was purchased from Biotrend (Schwabhausen, Germany). Caspase-3 colorimetric assay was from R&D Systems (Milan, Italy).

All other compounds were from Sigma-Aldrich Srl (Milan, Italy), unless otherwise specified.

Immunohistochemistry and immunocytochemistry

The surgical specimen was fixed in 10% neutral buffered formalin before being processed in paraffin. Immunohistochemical studies were performed using the streptavidin-biotin-peroxidase method (Ultra Vision kit; LAB VISION, Fremont, CA, USA) with diaminobenzidine as the chromogen and haematoxylin as the nuclear counterstain. A panel of antibodies against the following antigens was used: CgA (clone LK2H10; BioGenex; predilute; microwave antigen retrieval); synaptophysin (polyclonal; Cell Marque, Hot Springs, AR, USA; predilute; Ventana NexES immunostainer, Ventana Medical System Inc., Strasbourg, France; microwave antigen retrieval); NSE (clone E27; Cell Marque; predilute; Ventana NexES immunostainer; microwave antigen retrieval); SST (polyclonal; DAKO, Carpinteria, CA, USA;

dilution 1:4); insulin (polyclonal; DAKO; predilute); glucagon (polyclonal; DAKO; dilution 1:4); gastrin (polyclonal; BioGenex; dilution 1:150); and PP (polyclonal; DAKO; dilution 1:1000). Positive immunohistochemical controls included normal human tissues to which the appropriate antisera were added. Negative controls were performed by substituting the primary antibody with a non-immune serum of the same concentration.

Immunocytochemical localization of SSTR2 was performed in 4th passage SS-C cells grown on glass cover slips coated with poly-D-lysine (50 µg/ml). After washing with ice-cold Tris-buffered saline (TBS), cells were immediately fixed with 4% paraformaldehyde in PBS pH 7.2 for 20 min at room temperature. Fixed cells were washed with TBS (twice for 5 min each) and incubated at room temperature with 3% normal goat serum in TBS for 30 min. Receptors were detected by incubation at 4°C with SSTR2 antibody (1:1000 in TBS containing 0.2% Tween-20, 10% non-fat dry milk, and 10% glycerol). Cells were subsequently rinsed, twice for 5 min each, with TBS and incubated at room temperature for 30–60 min with FITC-conjugated goat anti-rabbit antibody (1:100). After two additional rinses with TBS (5 min each), the cover slips were mounted on glass slides with Mowiol/DABCO. The cells were imaged by epifluorescence microscopy. Controls were represented by the absence of primary antibody and by human skin fibroblasts.

Cultures of human somatostatinoma cells

Primary somatostatinoma tissue was surgically removed from jejunum, and metastatic tissue was removed from the liver. Tissue samples were obtained in accordance with a protocol approved by the Institutional Review Board for human studies. The patient provided informed consent as dictated by this protocol.

A fragment of the primary tumour and of one of the removed liver metastasis were collected in sterile conditions and transported to the laboratory. Tissue was minced into small fragments and digested overnight at 37°C with 0.3 mg/ml collagenase type II in Coon's modified Ham's F12 medium. Digested tissue fragments were gently centrifuged (500 g) transferred in Coon's modified Ham's F12 medium for mechanical dispersion and successively centrifuged at 500 g. The pellet was resuspended in Coon's modified Ham's F12 medium supplemented with 20% fetal calf serum (FCS) plus antibiotics, distributed in plastic culture dishes at a density of 5×10^5 cells/100 mm dish, and

cultured at 37°C in humidified 95% air/5% CO₂ atmosphere.

Phase contrast light microscopy was employed to examine the density and the morphological features of somatostatinoma cells.

Chromosome analysis

Cells were treated with colcemid at a final concentration of 10^{-6} M for 12 h before harvesting. After hypotonic treatment for 30 min at 37°C in 0.75% sodium citrate solution, the cells were fixed in methanol:acetic acid (3:1). Giemsa-stained specimens were examined for chromosome number, using more than 300 metaphase cells.

Determination of neuropeptides in culture media

To determine basal secretion of SST, CgA, glucagon, CGRP, calcitonin, insulin, C-peptide, gastrin, PP, GIP, CK, VIP, histamine, substance P and NSE, the cells after 7 days (1st passage), 40 days (5th passage), and 60 days (8th passage) in culture were detached from 100 mm culture dishes by trypsin solution (0.25% in PBS) treatment and plated into 35 mm dishes. After 24 h incubation cells were exposed for 30 min to Coon's modified Ham's F12 medium, the medium was then changed and the cells were finally incubated for 1 h in Coon's modified Ham's F12 medium. Then the medium was taken, centrifuged and stored at –80°C and the cells detached by trypsin and counted by haemocytometer. Negative controls were represented by Coon's modified Ham's F12 medium exposed for 1 h to plastic dishes alone. Positive controls were represented by Coon's modified Ham's F12 medium added with known quantities of human SST and exposed for 1 h to plastic dishes. Media from cell cultures were diluted from 1:10 to 1:1000 in PBS to allow better evaluation of peptide content. All samples from the same experiment were measured in the same assay. The results were expressed as pg/10⁵ cells per h. Absolute per cent variation from control data are reported as means ± s.d.

Effects of high K⁺ (50 mM), theophylline (10 mM) and octreotide (1 µM) on the release of SST from SS-C cells were determined in SS-C cells at the 1st passage. The cells were plated into 35 mm dishes (3×10^5 cells/dish) after trypsin treatment and a release experiment was performed on the 3rd day after plating. The cells were washed twice with PBS and incubated for 1 h in the presence or absence of test substances in the medium. Then, the medium was taken and stored at –80°C until analysis.

Table 1 Primers used in RT-PCR analysis

mRNA species	Upstream primer	Downstream primer	Fragment size (bp)	Tm (°C)
β -Actin	GACCTGACTGACTACCTCATGAA	CTTCATGATGGAGTTGAAGGTAG	303	64
IFN- γ	TGGCTTTTCAGCTCTGCATC	TCTCCACACTCTTTTGGATGC	251	60
IFN- γ -R1	GTCCTCAGTGCCTACACCAACTAA	CCACACATGTAAGACTCCTTC	594	62
IFN- γ -R2	CGAAGATTGCCTGTACAACGCA	GTCACCTCAATCTTTTCTGGAGGC	339	60

Oligonucleotides sequence is in 5'–3' orientation.

RNA preparation and RT-PCR

Total cellular RNA was extracted from primary cultures of SS-C cells at the 2nd passage by using RNeasy RNA Isolation Reagent, according to the manufacturer's instructions. Briefly, $1-2 \times 10^6$ cells were disrupted and homogenized in 1 ml denaturing solution. The lysate was then mixed with chloroform and centrifuged. After phenol/chloroform extraction, the RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and air-dried. The pellet was then dissolved in RNase-free water and stored at -80°C until required. Since no RNA isolation method is capable of consistently producing RNA free of contaminating DNA at the level of RT-PCR detection, in order to avoid any amplification from genomic contamination, the samples were pre-treated with 1 U of RNase A/DNase free (12.8 U/ μl) at 37°C for 30 min and heat-inactivated at 68°C for 10 min. The yield of RNA was calculated by spectrophotometry and to check RNA quality, 1 μg of each RNA sample was analysed by agarose gel electrophoresis and visualized on UV light.

IFN- γ , IFN- γ -R1, IFN- γ -R2 and β -actin (house-keeping gene) mRNA expression was evaluated by RT-PCR in SS-C cells at the 2nd passage and on previously collected human normal T lymphocytes, used as positive control for IFN- γ -R1 and IFN- γ -R2. For IFN- γ , which is not constitutively produced by T cells, an oligonucleotide (457 nucleotides, from position 135 to 591, referred to Genbank accession number NM_000619) probe was used. First-strand cDNA was synthesized with 1 μg total RNA using the Clone AMV First-Strand cDNA synthesis kit, according to the manufacturer's protocol. Briefly, 1 μg total RNA was reverse transcribed in 50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate, 4 $\mu\text{g}/\text{ml}$ BSA, 1 mM each deoxyribonucleoside triphosphate, 5 mM dithiothreitol, 2 U/ μl RNaseOUT recombinant ribonuclease inhibitor using 0.75 U/ μl cloned avian myeloblastosis virus (AMV) reverse transcriptase (RT), and 2.5 μM Oligo (dT)₂₀ as

first-strand cDNA primer in a total volume of 20 μl . RT reactions were carried out at 50°C for 60 min, followed by RT heat-inactivation at 85°C for 5 min and quick-chilled on ice. The resulting cDNA was stored at -20°C for subsequent amplifications. In the second step, IFN- γ , IFN- γ -R1, IFN- γ -R2 and β -actin expression was evaluated by PCR performed in separate tubes using primers specific for the genes of interest (Table 1) and puReTaq Ready-To-Go PCR beads. β -Actin was used as a constitutively expressed gene product for comparison of IFN- γ , IFN- γ -R1 and IFN- γ -R2 mRNA quality and concentration in each sample. Parallel RT-PCRs without added RT were performed to check genomic DNA contamination of samples. RT products (2 μl) were amplified with 2.5 U puReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 MgCl₂, 200 μM dATP, dCTP, dGTP and dTTP, 6 pmol of each pair of primers and sterile high-quality water to a final volume of 25 μl as follows: initial denaturation for 4 min at 94°C , 35 cycles of amplification, 30 s of denaturation at 94°C , different annealing temperatures for each pair of primers (IFN- γ /60 $^\circ\text{C}$, IFN- γ -R1/62 $^\circ\text{C}$, IFN- γ -R2/60 $^\circ\text{C}$, and β -actin/60 $^\circ\text{C}$), 30 s of extension at 72°C , and a final elongation step of 7 min at 72°C . Specific sets of upstream and downstream PCR primers for IFN- γ , IFN- γ -R1, IFN- γ -R2 and β -actin were designed on the basis of cDNA published sequences of Genbank Database: 5'-tggcttttcagctctgcatc and 3'-TCTCCACACTCTTTTGGATGC (amplified fragment of 251 bp) for IFN- γ -spanning exons 1–3, 5'-GTCCTCAGTGCCTACACCAACTAA and 3'-CCACACATGTAAGACTCCTTC (amplified fragment of 594 bp) for IFN- γ -R1 encompassing a region spanning exons 1–5, 5'-CGAAGATTGCCTGTACAACGCA and 3'-GTCACCTCAATCTTTTCTGGAGGC (amplified fragment of 339 bp) for IFN- γ -R2-spanning exons 2–4 and 5'-GACCTGACTGACTACCTCATGAA and 3'-CTTCATGATGGA-GTTGAAGGTAG (amplified fragment of 303 bp) for β -actin-spanning exons 4–5. Ten to 15 microlitres of RT-PCRs were electrophoresed with 50 bp and 100 bp

markers in 2% agarose gels stained with ethidium bromide and photographed. The specific RT-PCR products were excised, purified using a QUIAEX II kit and directly sequenced with an automated detection system (ABI-PRISM 3100; PE Applied Biosystems, Foster City, CA, USA) to confirm primer-specific amplification of cDNA.

The receptor expression pattern and the mRNA levels for the five SSTR subtypes (Hoyer *et al.* 1995) was analysed by real-time quantitative RT-PCR (Q-PCR) in SS-C cells at the 2nd passage, using a TaqMan 5'-exonuclease assay procedure. RT-PCR was performed as previously described with the RNA solution treated with DNase I to remove trace amount of genomic DNA contamination. Specific primers and probes for five SSTR genes were designed by Proligo Primers & Probes (Proligo LLC, Boulder, CO, USA) using the appropriate GenBank entries (sequences available on request). The homology between different subtypes was carefully examined and avoided. The lengths of all amplification products were between 60 and 186 bp. The primer specificity was tested by running a regular PCR for 40 cycles. After an initial denaturation at 94 °C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 60 °C and extension for 30 s at 72 °C. After a final extension for 10 min at 72 °C, 10 µl aliquots of the resulting PCR products were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide. The identity of the products was confirmed by direct sequencing using an ABI Prism 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's protocol. Several controls were included in the RT-PCR experiments. Since the SSTR genes are intron-less, to ascertain that no detectable genomic DNA was present in the total RNA preparation (even after DNase I treatment), the cDNA reactions were also performed without RT and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCRs of the SSTR subtypes, 0.001–0.1 µg human genomic DNA was amplified in parallel with cDNA samples. Q-PCR was performed for all five SSTRs, even if no expression of the SSTR3 and 5 subtypes could be detected in the cells we investigated by RT-PCR. In each experiment, standard curves for each primer set were included. Known amounts of dilutions of each PCR product were amplified in duplicate together with the unknown cDNA sample. A standard curve was constructed by plotting the threshold-cycle (Ct) vs the logarithm of the starting quantity (from

3×10 to 3×10^{10} molecules). By interpolation on these standard curves of the measured Ct of the unknown cDNA samples, the starting amounts in the cDNA sample were determined in duplicate. Reactions conditions were optimized until the s.d. of duplicate determinations of the Ct of standard curve samples was <3%. Levels of SSTRs genes in somatostatinoma cells are expressed as molecules of specific mRNA/µg total RNA and reported as means ± s.d. The primers and probe for SSTR2 mRNA quantification to use with the ABI Prism 7700 Sequence Detection System were selected by the proprietary software Primer Express, on the sequence NM 001050 (GeneBank). The forward primer corresponds to the region from base 448 to base 466 (sequence 5'-TCGGCCAAG-TGGAGGAGAC-3'). The reverse primer corresponds to the region from base 491 to base 510 (5'-AGAGACTCCCCACACAGCCA-3'). The internal oligonucleotide probe (CCGGACGG-CCAAGATGATCACC) is 5'-end FAM-labelled and 3'-end TAMRA-labelled. Four thousand nanograms of total RNA were reverse-transcribed by random examers, following the classic reverse transcription Perkin-Elmer protocol. The PCR mixture contains primers (200 mM each) and 200 nM of the TaqMan probe, with a final volume of 25 µl. Amplification and detection are performed with the ABI Prism 7700 Sequence Detection System with the following profile: one step at 50 °C for 2 min, one step at 95 °C for 10 min, and 40 cycles at 95 °C for 30 s, and 60 °C for 1 min. The amount of product was measured by interpolation from a standard curve of Ct values generated from known initial concentrations of RNA extracted from the neuroblastoma cell line CHP404, which over-expresses SSTR2 mRNA. One microgram of CHP404 RNA was reverse transcribed and cDNA was then serially diluted to obtain five standard solutions to be used in the PCR to generate the reference curve (Iguchi *et al.* 1990), with the first point corresponding to 2×10^9 molecules of SSTR2 mRNA/µg total RNA.

Whole-cell binding assay

SS-C cells were evaluated for the capability to bind to SST. SS-C cells at the 2nd passage were plated into four replicate wells of a 96-well polypropylene plates and grown to confluence. The medium was aspirated, and the cells were washed once with 50 mM Tris-HCl, pH 7.8, containing 1 mM EGTA, 5 mM MgCl₂ and 0.1% BSA (buffer A) and maintained in buffer A at room temperature for 30 min. This buffer was aspirated and replaced with buffer B (buffer A containing 1 mg/ml bacitracin). Test compounds were examined

over a range of concentrations from 0.01 to 10 000 nM at room temperature for 45 min in a final volume of 200 μ l using 0.1 nM 3-[¹²⁵I]iodotyrosyl²⁵-somatostatin-28 (Leu⁸,D-Trp²²,Tyr²⁵) ligand. The cells were washed three times with buffer A and then air-dried. The cells were solubilized by the addition of 25 μ l 1% SDS in 0.1 M NaOH to each well and then 200 μ l MicroScint-40 scintillation fluid. Radioactivity was determined in a scintillation counter. K_i values were calculated with the Cheng–Prusoff equation (Cheng & Prusoff 1973). K_d values for 3-[¹²⁵I]iodotyrosyl²⁵-somatostatin-28 (Leu⁸,D-Trp²²,Tyr²⁵) were determined from Scatchard plots of the saturation binding curves.

Cell growth and viability

Cell growth was measured by cell counting. For this purpose 3rd passage SS-C cells were plated at a density of 3×10^5 cells/35 mm dish under the indicated experimental conditions. After 96 h cells were detached with trypsin solution and the cell number was evaluated by a haemocytometer.

Cell viability was assessed by Trypan blue dye uptake/exclusion assay in SS-C cells after short-term exposure to various agents. Briefly, cells were exposed to a given stimulus for 30 min and then detached with trypsin solution. One drop of cell suspension was added to one drop of Trypan blue dye solution (0.3% in PBS) and blue stained vs unstained cells were counted and expressed as a percentage.

Measurement of telomerase activity

Telomerase activity in intact 2nd passage SS-C cells was measured using a modified non-radioactive telomerase repeat amplification protocol (Naasani et al. 1998, Falchetti et al. 1999). Telomere length was measured by a solution hybridization-based method (Gan et al. 2001). In brief, genomic DNA was isolated and 10 μ g DNA were digested at 37 °C overnight with 10 units each of HinfI/CpoI/HeaIII. The probe (TTAGGG)₄ was labelled with [γ -³²P]ATP with polynucleotide T4 kinase. Three nanograms of the probe were added to 2.5 μ g DNA solution. After denaturation at 98 °C for 5 min, hybridization was performed at 55 °C overnight. The resulting samples were electrophoresed on 0.7% agarose gel. After drying under vacuum without heating, the gel was exposed to a phosphorimage screen, and the results were analysed using the area-under-the curve method for the ImageQuant software. The point representing 50% of the area-under-the-curve was the mean telomere

length. Genomic DNA was extracted from homogenized human somatostatinoma cultured cells after different times of exposure to the telomerase inhibitor 3'-azido-3'-deoxythymidine (AZT). Human malignant mammary cancer (MCF-7) cells (ATCC; catalogue number HTB-22) were used as positive control (Soule & McGrath 1986, Kim et al. 1994). SS-C cells were treated with 100 μ M AZT for different times and total cells were collected and analysed for the mean telomere length.

Human 2nd passage SS-C cells were also evaluated for mutations of the *TP53* gene in exons 5–8. The *TP53* gene mutation screening kit was used to detect the presence of any kind of mutation in exons 5, 6, 7 and 8 of *TP53* gene, according to the manufacturer's instructions. Briefly 100 ng DNA were used in PCRs with five different pairs of primers (supplied in the kit) designed to span exons 5–8. MgCl₂ concentrations were 1.5 mM for primers A–C and E and 2 mM for primer D; annealing temperatures were 55 °C for primers A and E and 60 °C for primers B–D and PCR cycle steps were 2 min at 95 °C, 40 cycles 35 s at 94 °C, 40 s at the relative annealing temperature, 40 s at 72 °C and a final extension step at 72 °C. PCR products were analysed on a 2% agarose gel for specificity of reaction. Two microlitres of PCR product were added with 6 μ l denaturing loading buffer (95% v/v formamide, 10 mM EDTA, 0.1% basic fuchsin, 0.01% bromophenol blue), denatured at 95 °C for 5 min and loaded on a 12% acrylamide gel in TBE. Running conditions were 5 mA for 14–18 h in TBE 1 \times at 20 °C through water recycling. At the end of the run the gels were silver stained: 10% ethanol for 5 min, 1% nitric acid for 3 min, 2 mM silver nitrate for 20 min, three washes with 0.019% formaldehyde in sodium carbonate 0.28 M, 10% acetic acid for 2 min.

Flow cytometry

Antibody staining was performed on 1×10^6 4th passage SS-C cells for the conjugated primary antibody. Apoptosis was assayed with propidium iodide/annexin V staining. For propidium iodide/annexin V-FITC staining of apoptosis, cells were washed with annexin V FACS buffer (HBSS buffer with calcium, magnesium, sodium azide, and 0.5% BSA) and incubated on ice with 1:30 dilution of annexin V-FITC for 30 min and washed in FACS buffer. Prior to FACS analysis, propidium iodide at 5 μ g/ml was added to the cells and gently mixed. Stained cells were immediately analysed by FACS.

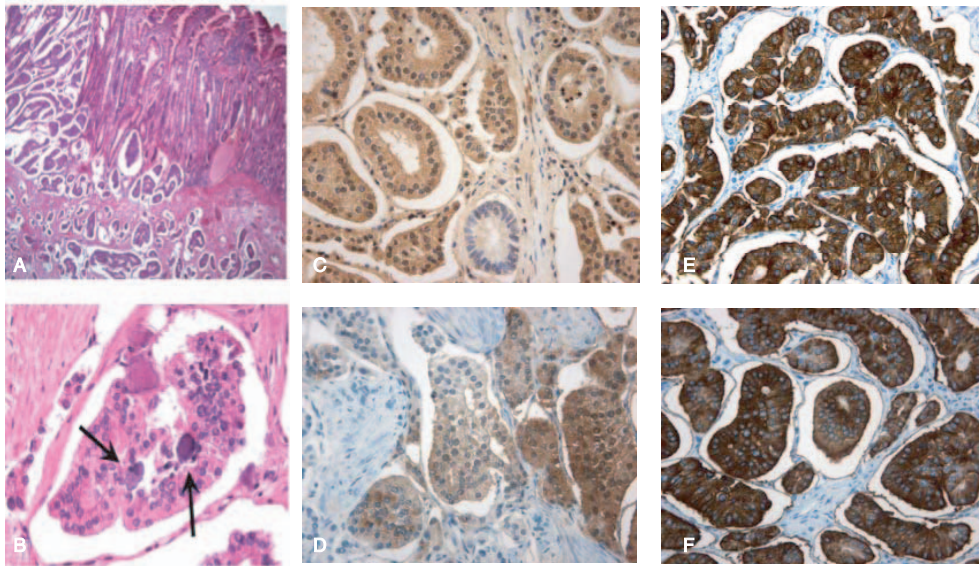


Figure 1 Microscopic view of the jejunal tumour with a vague acinar to ribbon-like pattern (A) (original magnification $\times 50$). High-power view of somatostatinoma revealing scattered psammoma bodies (B) (original magnification $\times 200$). Jejunal somatostatinoma tissue exhibited strong diffuse staining with somatostatin (C), NSE (D), CgA (E) and synaptophysin (F) (original magnification $\times 150$ for C and $\times 100$ for D–F).

Caspase activity

Apoptosis of 3rd passage SS-C cells was measured using a caspase-3 colourimetric assay with spectrophotometric quantification at 405 nm. Stimuli were added to the cells at the indicated concentrations and compared with control cultures (SS-C cells not exposed to any stimulus). Apoptosis was assessed following the manufacturer's protocol in cells cultured in six-well plates over a 24 h period. Cell protein content was assessed using a detergent-compatible protein assay reagent, based on the Lowry procedure (Lowry *et al.* 1951) and lyophilized bovine plasma gamma globulin was used as standard.

Statistics

All the experiments were carried out in triplicate and data expressed as means \pm s.d. of triplicate experimental points. Statistical differences were analysed using one-way ANOVA and significance was evaluated by standard Chi-square test by using Statistica 5.1 (Statsoft Inc., Tulsa, OK).

Results

Patient's clinical features

The high circulating levels of SST (3233 pg/ml; normal value: ≤ 37 pg/ml) and NSE (17.7 ng/ml; normal value: 0–13 ng/ml) confirmed the presence of metabolic

somatostatinoma. A genetic test for *MEN1* gene mutations was negative. CT evidenced the presence of hepatic lesions. An MRI demonstrated that the hepatic results were compatible with secondary lesions. Pancreatic echoendoscopy did not evidence lesions of the pancreatic, gastric and duodenal levels. An Octreoscan (scanning agent was 111 Indium-diethylene-triamine pentaacetic acid-octreotide) [111 In-DTPA octreotide] was negative, probably due to a saturation of the receptor sites by the high levels of circulating SST.

Pathology of tumour tissues

Microscopically, the tumour cells, small to medium-sized round cells with clear large cytoplasm, were arranged in a trabecular or ribbon pattern separated by delicate fibrovascular stroma. Scattered psammoma bodies were identified within the tumour (Fig. 1). Immunohistochemically, the cytoplasm of the neoplastic cells from the primary jejunal lesion and from the liver metastases (data not shown) was strongly positive for SST, CgA, NSE and synaptophysin (Fig. 1). Tumour cells showed no positive staining for insulin, glucagon, gastrin and PP.

Cell cultures

Cells obtained from the primary tumour and from one of the liver metastases were cultured in growth medium

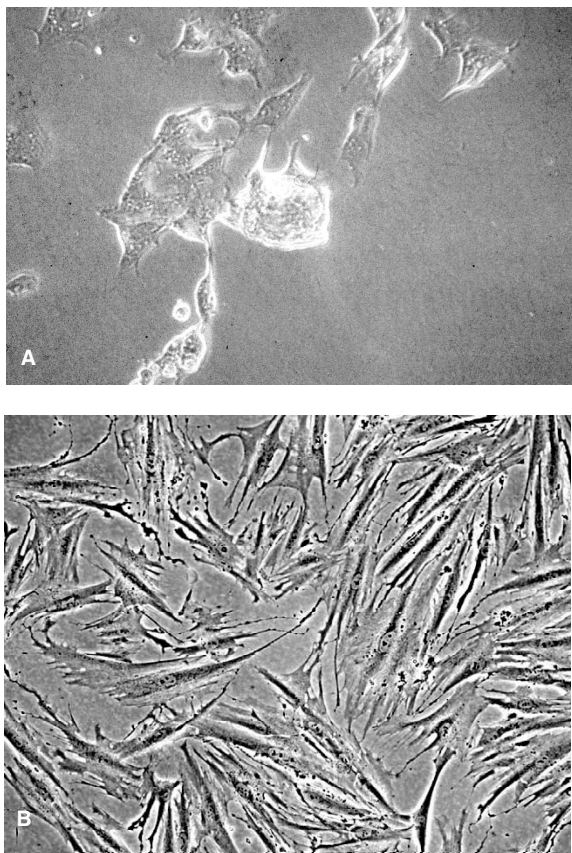


Figure 2 Contrast phase image of (A) primary cultures of somatostatinoma (original magnification $\times 200$) and of subconfluent SS-C cells at the 3rd passage (original magnification $\times 100$).

and analysed at different times for morphological, growth and differentiative properties. Cell cultures obtained from the liver metastasis were not successful, as they did not adhere to the culture dish. Conversely, primary cultures from the primary somatostatinoma tissue were characterized by clusters of epithelial-like cells from which elongated cells originated and colonized the culture dish (Fig. 2). These cells, obtained in very large quantity out of the primary tumour and indicated as SS-C cells, grew as a firmly attached monolayer, showing a homogeneous phenotype with a neuronal-like appearance (Fig. 2). Weekly passage of SS-C cells made it possible to obtain well-characterized cultures to be frozen and used for functional and growth studies. The population doubling-time of SS-C cells calculated from the growth curve ranged from 3.2 days at the 1st passage to 4 days at the 5th serial passage and to 5.8 at the 12th serial passage. The chromosome counts of 100 metaphase

Table 2 Basal secretion rates of SST, other gastroenteropancreatic hormones, CgA, and NSE by SS-C cells at 7 days (1st passage), 40 days (5th passage) and 60 days (8th passage) in culture

Product	Basal secretion ^a (pg/10 ⁵ cells/h)		
	7 days (1st passage)	40 days (5th passage)	60 days (8th passage)
SST	3723 \pm 440	2825 \pm 360	1641 \pm 318
CgA	40450 \pm 813	28511 \pm 2077	18640 \pm 2880
Glucagon	25 \pm 2	15 \pm 3	N.D.
CGRP	18 \pm 3	N.D.	N.D.
Calcitonin	8 \pm 0.5	N.D.	N.D.
Insulin	N.D. ^b	N.D.	N.D.
C-Peptide	N.D.	N.D.	N.D.
Gastrin	N.D.	N.D.	N.D.
PP	N.D.	N.D.	N.D.
GIP	N.D.	N.D.	N.D.
CK	N.D.	N.D.	N.D.
VIP	N.D.	N.D.	N.D.
Histamine	N.D.	N.D.	N.D.
Substance P	N.D.	N.D.	N.D.
NSE	N.D.	N.D.	N.D.

^aMeans \pm s.d. of three replicate dishes in two independent experiments.

^bN.D., not detectable.

cells revealed the modal peak to be 46, with only few cells showing some deviation from this diploid mode. SS-C cells did not show changes in morphological characteristics up to the 12th serial transfer, with major signs of senescence observed afterwards. For this reason in this study SS-C cells were used between the primary culture and the 5th passage.

Confluent cultures of SS-C cells were tested for hormonal production. Similar to what was observed in tissue immunohistochemistry, SS-C cells at the 1st passage (7 days), at the 5th passage (40 days), and at the 8th passage (60 days) exhibited the ability to secrete SST into the culture medium (Table 2). The molarities of SST in the culture medium reached a maximum of 50 nM. High amounts of CgA were released into the culture medium by SS-C cells at the 1st passage, with half of the values found after 8 passages (Table 2). Measurable levels of glucagon, CGRP and calcitonin were detected in 1st passage SS-C cells, while this characteristic was lost in long-term cultures (Table 2). No release of insulin, C-peptide, gastrin, PP, GIP, CK, VIP, histamine, substance P, and NSE was detected in culture media of primary cultures and long-term cultures of SS-C cells. None of the neuropeptides tested was measurable in the Coon's modified Ham's F12 medium used for the incubation. The addition of

known concentrations of SST to the culture medium allowed 92% recovery.

As shown in Fig. 3, after 1 h incubation, 50 mM K⁺ caused a 6-fold increase of SST secretion by SS-C cells at the 1st passage. Significant stimulation (8-fold) of SST secretion was seen in the presence of 10 mM theophylline (Fig. 3). Octreotide (1 μM) was able to decrease by 58% the release of SST from 1st passage SS-C cells (Fig. 3). Basal values of SST release in the controls were 4100 ± 640 pg/10⁵ cells.

Characterization of expression of SSTRs, IFN-γ and IFN-γ-Rs

In the qualitative analysis of SSTRs, IFN-γ, IFN-γ-R1 and IFN-γ-R2 expression no PCR products were detected in samples without RT or in the absence of cDNA template. No expression of the SSTR3 and 5 subtypes could be detected in SS-C cells at the 2nd passage by RT-PCR (Fig. 4A). Q-PCR performed for

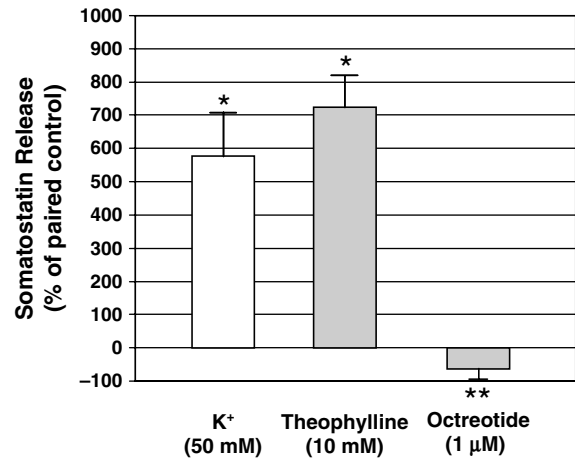


Figure 3 Effect of 50 mM K⁺, 10 mM theophylline and 1 μM octreotide on SST secretion by SS-C cells at the 1st passage. SS-C cells were incubated in triplicate for 1 h in the presence of the different stimuli. Columns, means ± s.d. of triplicate samples. **P* < 0.05 and ***P* < 0.01 (vs untreated cells).

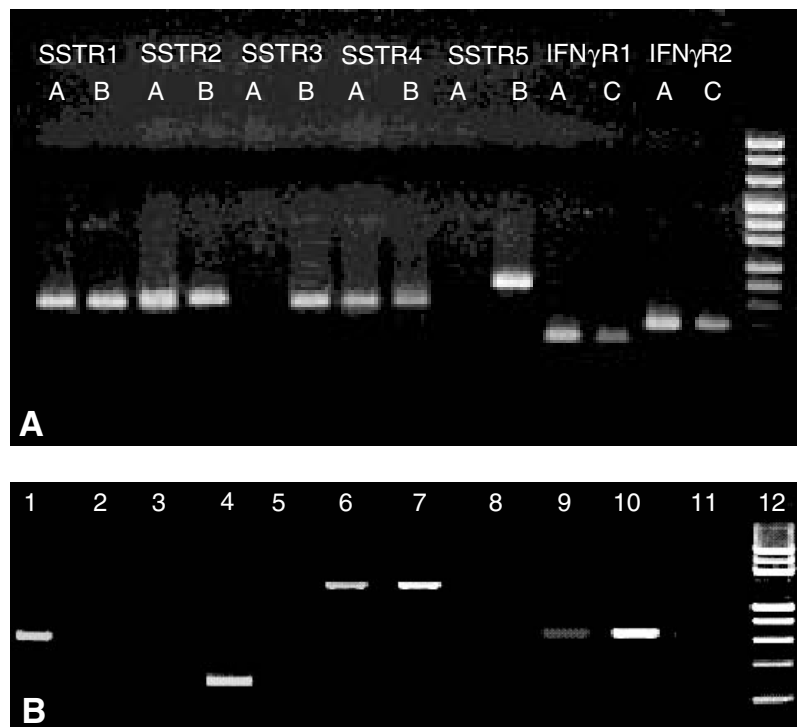


Figure 4 (A) Expression of mRNA for SSTR subtypes and IFN-γ, IFN-γR1 and IFN-γR2 in SS-C cells. Real-time RT-PCR efficiencies confirmed by a regular RT-PCR. Total RNA was extracted from SS-C cells at the 2nd passage. PCR products of SSTR 1–5 and IFNγR-1 and 2 were separated on 2% agarose gel and stained with ethidium bromide. Molecular weight marker is DNA Molecular Weight Marker VIII (Roche); 'A': somatostatinoma sample; 'B': genomic DNA as positive control; 'C': T lymphocytes as positive control. RT-PCR analysis of each SSTR was performed at least twice with identical results. (B) Expression of mRNA in SS-C cells by conventional RT-PCR using primer set shown in Table 1. SS-C cell β-actin is shown in lane 1; lanes 2, 5 and 8 represent negative controls (PCR without template). In lanes 3, 6 and 9, IFN-γ, IFN-γ-R1 and IFN-γ-R2 are respectively depicted. Lanes 4, 7 and 10 contain positive controls (normal human T lymphocytes). In lane 12 a 100 bp DNA ladder (size range 100–1000 bp) is shown.



Figure 5 Immunocytochemical localization of SSTR2 in SS-C cells at the 4th passage. A specific SSTR2 antibody evidenced a positive diffuse cytoplasmic staining. Original magnification $\times 100$.

all five SSTRs confirmed these findings and no Ct was observed for SSTR3 and 5. Expression levels of SSTR1, 2 and 4 genes in SS-C cells expressed as molecules of specific mRNA/ μg total RNA reported as mean \pm s.d. were: SSTR1, $(2.19 \pm 0.05) \times 10^{12}$; SSTR2, $(1.39 \pm 0.04) \times 10^8$; and SSTR4, $(7.82 \pm 0.17) \times 10^6$. SS-C cells at the 2nd passage showed SSTR2 gene expression, with a mean value of 3.48×10^6 molecules of SSTR2 mRNA/25 ng total RNA, corresponding to 1.39×10^8 molecules of SSTR2 mRNA/ μg total RNA (data not shown).

RT-PCR analysis of IFN- γ , IFN γ -R1, IFN γ -R2 and β -actin (housekeeping gene) mRNA in 2nd passage SS-C cells is shown in Fig. 4B. A higher expression of both IFN- γ -R1 and -R2 mRNAs was observed in SS-C cells compared with the positive control (normal human T lymphocytes), whereas IFN- γ was not expressed in SS-C cells (Fig. 4B).

Immunocytochemical localization of SSTR2 in SS-C cells at the 4th passage showed a diffuse cytoplasmic staining (Fig. 5). No labelling was observed in the absence of the primary antibody and in human skin fibroblasts (data not shown). In SS-C cells at the 2nd passage the apparent dissociation constants for SST-28 and octreotide were 0.4 and 0.7 nM respectively (data not shown).

Telomere length and modulation by a telomerase inhibitor

Human 2nd passage SS-C cells showed a telomere length of 1588 ± 179 bp, with no mutations in exons 5–8 of the *TP53* gene (data not shown). The telomerase

inhibitor, AZT, at a concentration of 0.1 mM inhibited in a time-dependent manner telomerase activity in 2nd passage SS-C cells, with maximal effect ($49 \pm 9\%$) at 72 h (data not shown).

Antiproliferative activity of SST-28, octreotide, IFN- γ and AZT on human SS-C cells

Having established functional SST-28 and IFN- γ receptors and AZT signalling, we evaluated the effects of SST-28, octreotide, IFN- γ and AZT on growth of 3rd passage SS-C cells cultured in medium containing 10% FCS. SST-28 and octreotide inhibited SS-C cell proliferation in a dose-dependent manner with maximal effect at micromolar doses (Fig. 6). Similarly, the antiproliferative action of IFN- γ was dose-dependent, with a maximal inhibition to $25 \pm 4.2\%$ of control observed at a concentration of 500 IU/ml (Fig. 6). AZT inhibited SS-C cell proliferation at a range of concentrations from 0.01 to 1.0 mM, with maximal effect at 1.0 mM (Fig. 6). In the same experimental conditions the combined inhibitory action of $1 \mu\text{M}$ SST-28 with 500 IU/ml IFN- γ and 1 mM AZT was additive, while octreotide was inert in the presence of the SST-28 (Fig. 7). None of the stimuli were able to affect SS-C cell viability as assessed by the Trypan blue exclusion test after 30 min exposure to the stimuli (data not shown).

SST-28, octreotide, IFN- γ and AZT induce apoptosis of SS-C cells

SS-C cells at the 4th passage were cultured for 48 h in the presence of increasing concentrations of SST-28 (0.001–1.0 μM), of octreotide (0.001–1.0 μM), of IFN- γ (0.5–500 IU/ml) and of AZT (0.001–1.0 mM) and then apoptosis was measured by annexin V flow cytometry. All three substances induced apoptosis in a dose-dependent manner in SS-C cells (Fig. 8).

Twenty-four hour exposure to SST-28 and octreotide at $1 \mu\text{M}$ increased apoptosis in the 4th passage SS-C cells as determined by caspase-3 activity, by respectively 580% and 567% compared with control cells ($P < 0.001$) (Table 3). Caspase-3 activity was also significantly increased in cells exposed to IFN- γ (500 IU/ml) and AZT (1 mM) (Table 3).

Discussion

Somatostatinoma is a rare disorder whose diagnosis at a metastatic stage is not infrequent and the treatment with non-surgical anticancer therapies is doubtful. Moreover, the low percentage of proliferating tumour

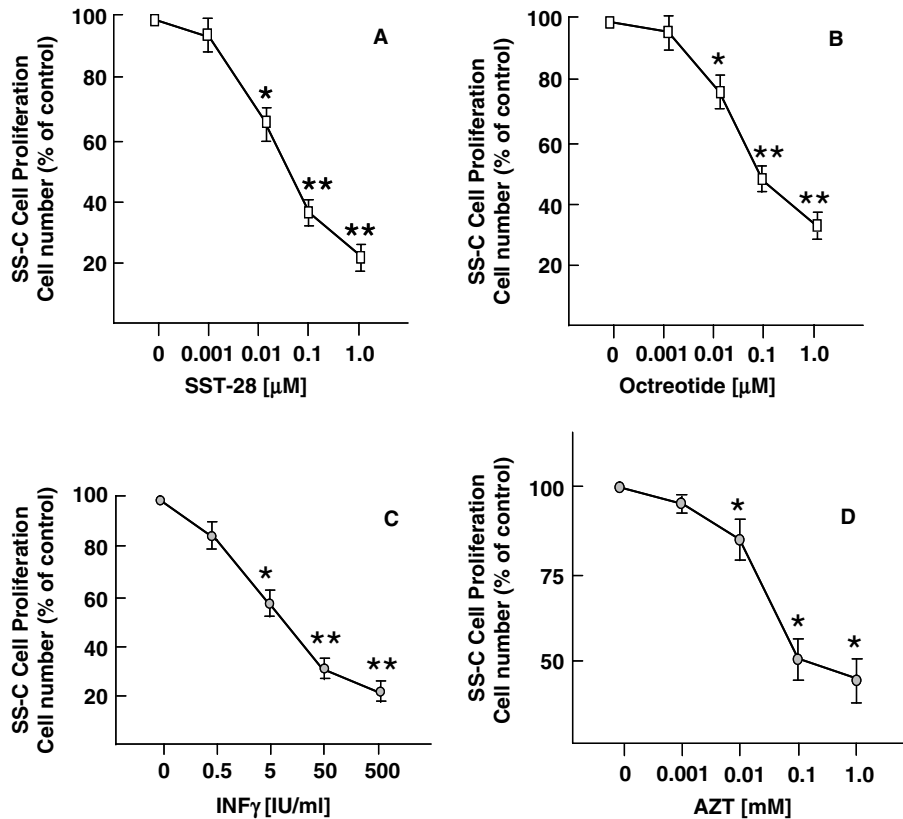


Figure 6 SST-28 (1 μM), octreotide (1 μM), IFN- γ (500 IU/ml) and AZT (1 mM) inhibited proliferation of 2nd passage SS-C cells cultured in the presence of 10% FCS. Results are means \pm s.d. of three experimental points. * P <0.05 and ** P <0.01 (vs untreated cells).

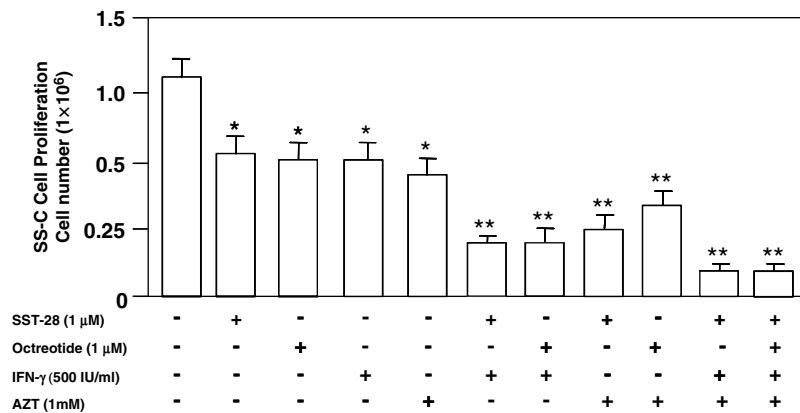


Figure 7 SST-28 (1 μM), octreotide (1 μM), IFN- γ (500 IU/ml) and AZT (1 mM) inhibited proliferation of 3rd passage SS-C cells cultured in the presence of 10% FCS. The combined presence of SST-28, IFN- γ and AZT caused an additive effect, while octreotide was inert in the presence of SST-28. Results are means \pm s.d. of three experimental points. * P <0.05 and ** P <0.01 (vs untreated cells).

cells limits the use of conventional chemotherapy, while the need for therapy rather arises from the presence of an SST hypersecretion syndrome.

Currently, available systemic treatment options for somatostatinoma suffer from failure to achieve convincing tumour regression. For all these reasons the

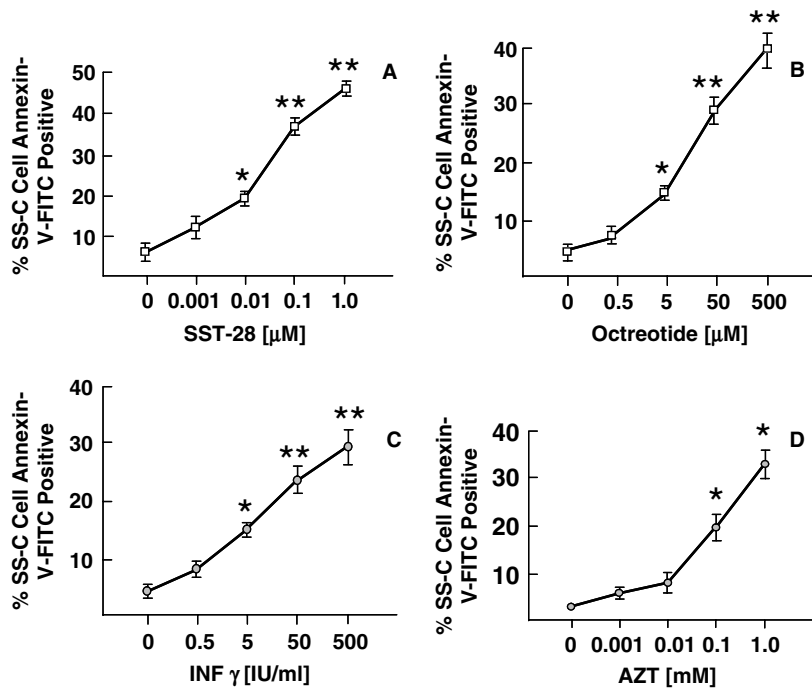


Figure 8 SST-28 (A), octreotide (B), IFN- γ (C) and AZT (D) induced apoptosis in a dose-dependent manner in 4th passage SS-C cells, as measured by annexin V flow cytometry. Results are means \pm s.d. of three experimental points. * P <0.05 and ** P <0.01 (vs untreated cells).

Table 3 Effect of SST-28, octreotide, IFN- γ and AZT on caspase-3 activity (optical density at 405nm/mg protein) in SS-C cell cultures at the 4th passage

	Caspase-3 activity* (OD/mg protein)	P value
Control	0.19 \pm 0.01	–
SST-28 (1 μ M)	1.10 \pm 0.12	<0.001
Octreotide (1 μ M)	1.07 \pm 0.09	<0.001
IFN γ (500 IU/ml)	0.61 \pm 0.07	<0.01
AZT (1 mM)	0.78 \pm 0.09	<0.01

*Results are expressed as means \pm s.d. of six experimental points.

development of well-characterized antiproliferative strategies appears mandatory.

The present report describes the long-term culture of a human cell line, SS-C, from a patient diagnosed as having somatostatinoma of the jejunum. The characteristics of SS-C cells are high production of SST and of CgA, a neuronal-like morphology and growth as a monolayer. SST and CgA expression were evidenced also in the somatostatinoma tissue, that, differently from cultured cells also expressed NSE. SS-C cells also responded to depolarizing and cAMP-dependent stimuli, as shown in a previous model of human

SST-secreting cells in continuous culture, the QGP-1 cell line (Detjen *et al.* 2002). The latter cell model was characterized by the production of SST, PP and CEA (Detjen *et al.* 2002). However, the tumour from which QGP-1 cells were derived was not indicated as a somatostatinoma, but as a ‘neuroendocrinepancreatic tumour’ (Detjen *et al.* 2002). In addition, in SS-C cells the SST analogue, octreotide, was able to significantly inhibit SST release in culture. The rarity of somatostatinoma cell cultures makes the SS-C cell line a unique model for evaluation of both molecular marker expression and *in vitro* effects of molecules active on expressed targets. Here we provide evidence for control of human SS-C cell proliferation via growth inhibition and apoptotic cell death by SST-28, octreotide, IFN- γ and an inhibitor of telomerase activity, AZT.

SS-C cells expressed mRNA for type 1, 2 and 4 SSTR, confirming that multiple SSTR subtypes are coexpressed in the same cell. The expression of SSTR subtypes in SS-C cells diverges from the pattern described for somatostatinoma tissues in a previous report of cases (Papotti *et al.* 2002). Such a difference can be interpreted as a cell-specific phenotype, as supported by data concerning the cell-specific localization of the SSTR subtypes (Mezey *et al.* 1998). However, the expression of type 2 SSTR and its

cytosolic distribution are in agreement with immunohistochemical results on somatostatinoma tumour tissues (Reubi *et al.* 2000, Papotti *et al.* 2002, Liu *et al.* 2003). The expression of SSTs in human somatostatinoma cells represents a logical basis for the use of native SST and/or its analogues in the control of differentiative and proliferative features of this tumour.

The use of the pan-receptor agonist SST-28 showed that the natural hormone inhibits proliferation of growing human SS-C cells, while inducing cellular apoptosis. Interestingly, the maximal *in vitro* effect of SST-28 was obtained at doses 10- to 100-fold higher than the concentrations of the hormone released into the culture medium. The well-characterized antiproliferative activity of SST-28 on other endocrine tumours (Arnold *et al.* 1996, Florio *et al.* 2003) opens interesting possibilities in the medical treatment of this rare endocrine malignancy. Natural SST-28 is not suitable for long-term clinical application, but in clinical practice SST analogues that show a preferential affinity for SSTR2 and SSTR5, with medium affinity to SSTR3 (octreotide and lanreotide) are available (Yang *et al.* 1998). Previous *in vivo* studies showed that Octreoscan-positive somatostatinomas respond to octreotide therapy with decrease in the plasma levels of SST (Angeletti *et al.* 1998). As for other targets also in somatostatinoma tissue octreotide exerted its biological actions by inhibiting both hormone secretion and cell proliferation, and via induction of apoptosis. Interestingly, as the binding affinity and biological actions of SST-28 and octreotide are superimposable in SS-C cells, the SSTR2 molecules appear responsible for their biological effects on this cell model, including apoptosis signalling. Our results add new information on the *in vitro* direct effects of SST on somatostatinoma cells, opening the possibility of the need for testing somatostatinoma tissue for expression of SSTs even in Octreoscan-negative patients.

SS-C cells also express IFN- γ receptors. IFN- γ is a cytokine which also acts as a key regulator of proliferation, differentiation and survival, mainly via the inhibition of G1 or S-phase cell cycle progression (Platanias & Fish 1999, Sangfelt *et al.* 2000) and the induction of apoptosis (Tamura *et al.* 1996). Interestingly, the human SST-secreting cell line QGP-1, obtained from a neuroendocrine pancreatic tumour, expresses IFN- γ receptors and IFN- γ acts as a potent proapoptotic stimulus in these cells (Detjen *et al.* 2002). The present findings of antiproliferative and proapoptotic actions of IFN- γ in cells derived from a human somatostatinoma tissue provide important clues for therapeutic strategies in this endocrine tumour.

Moreover, SS-C cells express telomerase activity, offering an additional target for pharmacological intervention in somatostatinoma. AZT is a molecule commonly used as an antiviral agent and also showing antitumour activity, probably via inhibition of chain elongation at the telomeric ends of chromosomes in cancer cells (Furman *et al.* 1986, Furman & Barry 1988, Fischl *et al.* 1989, Posner *et al.* 1990, 1992, 1993, Beitz *et al.* 1992, Anand *et al.* 1995, Strahl & Blackburn 1996, Furman *et al.* 2000). This study indicates that *in vitro* exposure of SS-C cells to AZT results in inhibition both of telomerase and of cell proliferation. We also found that AZT induced an apoptotic cell death in SS-C cells, as previously shown in Herpes virus-associated lymphomas (Lee *et al.* 1999) and in parathyroid carcinoma (Falchetti *et al.* 2005).

Finally, the inhibitory effect of SST-28, IFN- γ and AZT on SS-C cell proliferation was additive, opening the possibility of the use of these agents in *in vivo* treatment of somatostatinoma.

In summary, these results characterize SST-28, octreotide, IFN- γ , and AZT as antiproliferative and proapoptotic stimuli in human somatostatinoma cells in long-term culture. These findings have a number of implications. First, this delineation in a human cellular model provides important clues on targets that might be analysed *in vivo* and/or *in vitro* to predict therapeutic responsiveness in somatostatinoma patients. Secondly, the use of anticancer agents that induce tumour apoptosis would probably lessen patient morbidity from complications of tumour lysis. Thirdly, based on these results, the potential of combined treatment with active compounds in experimental biotherapeutic treatment of somatostatinoma should be explored.

Acknowledgements

This paper has been supported by AIRC 2000 (to M L B), by Cofin MIUR 2003 (to F T) and by Fondazione Ente Cassa di Risparmio di Firenze (to M L B). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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