Somatostatin receptor gene expression and inhibitory effects of octreotide on primary cultures of orbital fibroblasts from Graves’ ophthalmopathy

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ABSTRACT

To explore the mechanism underlying the effects of the somatostatin (SST) analogue octreotide in Graves’ ophthalmopathy (GO), we investigated the expression of SST and of SST receptor (sst1–5) genes in primary cultures of fibroblasts established from retroorbital tissue of GO patients and of control subjects. We determined also SST specific binding sites by competitive binding of [125I-Tyr11]SST-14 and the effect of octreotide on cell growth, cAMP accumulation, Bcl-2 intracellular levels and apoptosis in GO fibroblast primary cultures. All primary cultures expressed the SST gene transcript and one or more ssts that have a high affinity for the two analogues (class 1 sst). The sst2 transcript was found in nine, sst3 in five and sst5 in eight out of ten GO cell cultures. Sst2 was detected in all six, and sst3 in four out of the six control cell cultures. Sst4 was absent from all samples, and sst1 was found only in six out of the ten GO samples. SST-14 and octreotide inhibited the binding of [125I-Tyr11]SST-14 with a half-maximal inhibition of binding (IC50) of 0·80 ± 0·37 and 33·7 ± 33·1 nmol/l respectively in GO cell cultures, and with an IC50 of 0·9 and 1·5 nmol/l in control cultures. Octreotide (10−6 and 10−7 M) significantly decreased (P < 0·001) forskolin-induced but not basal cAMP accumulation; at both doses for 72 h it inhibited cell growth (20 and 55% respectively), and induced apoptosis (20 and 40%), and abolished Bcl-2 protein in cell lysates. In conclusion, SST and sst transcripts are expressed and functional in cultured retroorbital fibroblasts. The presence of class 1 sst in GO tissue and the inhibition exerted by octreotide on retroorbital cell growth and activity in vitro may account for the effects of SST analogue administration in vivo in GO.

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INTRODUCTON

Severe ophthalmopathy complicating autoimmune thyroid diseases requires aggressive treatments (i.e. immunosuppressive therapy, radiotherapy or surgical orbital decompression), but a substantial number of patients do not respond to these treatments or suffer major side-effects (Burch & Wartofsky 1993, Gorman et al. 1994, Bartalena et al. 1997). Several clinical reports have shown that two long-acting somatostatin (SST) analogues, octreotide and lanreotide, have beneficial effects in Graves’ ophthalmopathy (GO) (Chang et al. 1991, Krassas et al. 1995, 1997, Kung et al. 1996, Ozata et al. 1996), although the number of patients enrolled in the studies so far reported is too limited to allow a definitive conclusion.

The possible mechanism of action of SST analogues on GO remains unexplained (Ozata et al. 1996, Bartalena et al. 1997). Accumulation of radiolabelled SST analogue in retroorbital tissue has been demonstrated in vivo by 111In-DTPA-d-Phe1-octreotide (Octreoscan) scintigraphy (Chang et al. 1991, Postena & Krenning 1994, Kahaly et al. 1995, Krassas et al. 1995, Colao et al. 1998), suggesting the presence of specific binding sites for octreotide on the orbit components. It is not clear whether octreotide binds to retroorbital fibroblasts...

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or to infiltrating immune cells. Neither is it known whether SST analogues act solely by inhibiting local immune cells or whether they also exert an effect directly on fibroblast growth and activity. SST affects target tissues through specific membrane receptors, encoded by five genes (sst1-5), that display different affinities for the SST analogues used in clinical practice (Reisine & Bell 1995, Patel 1997). Octreotide and lanreotide bind with an intermediate to high affinity to sst2, sst4 and sst5, which belong to the class 1 sst family. They do not show significant binding to sst1 and sst4, i.e. the class 2 sst family (Patel 1997). Functional ssts have been detected in activated human peripheral lymphocytes and in pathological and non-pathological lymphoid tissues and may play a negative regulatory role in the immune response (Lamberts et al. 1991, 1996, Van Hagen 1996). In the present study, we investigated the expression of SST and SST receptor genes in primary fibroblasts derived from retroorbital tissues of GO patients, and the effects in vitro of SST and octreotide on these cells.

MATERIAL AND METHODS

Origin of tissues and cell cultures

Retroorbital connective tissue was obtained from ten patients with GO during orbital decompression surgery. The diagnosis was based on endocrinological and ophtalmological criteria, including laboratory determination of hormones and antibodies and imaging studies (ultrasonography, computerized tomography or magnetic resonance scan) of orbits. The patients (seven females, three males; aged 35–68 years), euthyroid at the time of surgery, had never been treated with steroids or had been off therapy for over 6 months. Control orbital tissues were obtained from six subjects (four females, two males; aged 30–67 years) undergoing eye surgery for trauma, osteoma or strabismus. No control subject had a history of autoimmune or thyroid diseases, or trauma, osteoma or strabismus. No control subject had a history of autoimmune or thyroid diseases, or was affected by orbital inflammatory conditions. Informed consent was obtained from all patients to use orbital tissue samples for in vitro study. Tissue explants were minced and placed directly in plastic culture dishes for incubation with the appropriate medium in a humidified incubator (37 °C, 5% CO2). Fibroblasts were allowed to proliferate in modified Eagle’s medium (MEM) supplemented with 1-glutamine, 10% (v/v) foetal bovine serum and antibiotics (Gibco-BRL, Life Technologies, Milan, Italy). Four cell strains from normal and four from GO tissue specimens were used in the experimental protocols, which were repeated at least three times.

mRNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cultures at early passages (first to fourth). Total RNA was recovered with an RAZOL B kit (Cinna/Biotec Laboratories, Houston, TX, USA). Residual DNA was removed by RNase-free DNase I treatment (Promega, Florence, Italy). RT-PCR was carried out as previously described (Sinisi et al. 1997). RNAs were reversely transcribed using 5 µg total RNA after annealing with 0·2 nM oligo (deoxythymidine) for priming of cDNA in the presence of reverse transcriptase (Superscript, BRL, 200 U) at 37 °C for 1·5 h. The reaction was stopped by incubation at 95 °C for 5 min. To obtain a negative control for the amplification reactions, we carried out an RNA transcription without reverse transcriptase. Six hundred nanograms cDNA obtained by reverse transcription of RNAs were amplified in a total volume of 50 µl Tris–HCl 10 mmol, 1·5 mmol MgCl2 and 50 mmol KCl pH 8·3, 100 ng of primers, deoxynucleotides triphosphate 0·2 mmol and 2·5 U Taq DNA polymerase (Boehringer, Mannheim, Germany). A DNA thermal cycler (Perkin-Elmer/Cetus, Milan, Italy) was used for the reaction. The reaction was started by a 3 min denaturation at 95 °C; this was followed by 40 cycles of 1 min annealing at 60 °C, a 2 min extension at 72 °C and a 30 s denaturation at 95 °C. One hundred nanograms glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were added to each PCR reaction (25 cycles), as internal control, and the 876 bp product of GAPDH was detected in each PCR reaction. The number of cycles was chosen in the middle of the exponential phase of the reaction, separately for each type. To establish the number of cycles, GAPDH was amplified at 15, 22, 32 and 40 PCR cycles, and sst1-5 and SST were subjected to 25, 32, 40 and 45 amplification cycles (data not shown). The 5′-3′ oligonucleotides for sst1, sst2, sst3, sst4 and sst5, SST and GAPDH are shown in Table 1. The PCR products were analysed by electrophoresis on a 1·5% agarose gel and by comparing their size with the size expected from the gene sequence. The identity of the products from representative reactions was confirmed by direct sequencing of PCR products.

The treatment with DNase and the coamplification of the GAPDH gene containing introns excluded genomic DNA contamination. Moreover, no products were detected in control amplifications.

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performed without cDNA (negative control, data not shown).

**Binding studies**

Cells from an early passage (first or second) were cultured in 35 mm diameter dishes in MEM supplemented with 10% foetal calf serum (FCS) at a density of 4 × 10^5/dish. The cells were washed twice with Krebs/Hepes buffer, binding was performed at 25 °C for 120 min in a final volume of 1.5 ml Krebs/Hepes buffer (pH 7.4) containing 15 mg/ml BSA, 0.3 mg/ml soybean trypsin inhibitor, 0.5 mg/ml bacitracin and 30 pM [¹²⁵I-Tyr¹¹]somatostatin (Amersham, Milan, Italy). Non-specific binding was determined in the presence of 1 μM somatostatin-14 (SST-14) (Sigma Peptides, Milan, Italy) or SMS 20–995 (Sandostatin; Novartis, Milan, Italy). The cells were then washed and lysed in 0.1 M NaOH and specific binding was quantified. In ligand competition experiments, cells were incubated under the same conditions with 30 pM [¹²⁵I-Tyr¹¹]somatostatin and varying concentrations of unlabelled peptide analogues.

**Cell proliferation assay**

Cell proliferation was evaluated with the tetrazolium salts (MTT) method. The Cell Proliferation assay (Boehringer-Mannheim) is a colorimetric assay (MTT-based) for the non-radioactive quantification of cell proliferation and viability. MTT is useful for the quantification of viable cells, because only metabolically active cells cleave it to form a formazan dye (UV absorbance spectrum is between 550 and 600 nm). Cells were seeded in microtitre plates in a final volume of 100 μl complete culture medium at a concentration of 2 × 10^3 cells/well and grown for 24 h at 37 °C in 5% CO₂. Cells, starved for 24 h in MEM without FCS, were incubated in 1% FCS-supplemented medium with octreotide 10⁻⁶, 10⁻⁷ and 10⁻⁸ M or solvent (control cells) for 96 h. Then 10 μl MTT solution were added to each well and plates were incubated for 4 h. Ten microlitres of solubilization solution were added to each well and plates were kept overnight in the incubator. Absorbance was read at 550 nm using a microtitre plate reader.

**Apoptosis detection**

The In Situ Cell Death Detection Kit (Boehringer Mannheim, TUNEL) was used to detect apoptosis and to quantify DNA strand breaks in individual cells. The cell monolayers were grown directly on sterilized slides (Superfrost; Carlo Erba, Milan, Italy), starved for 24 h in MEM without FCS, and then incubated in 1% FCS-supplemented medium with octreotide 10⁻⁶, 10⁻⁷ and 10⁻⁸ M or solvent (control cells) for 96 h. The slides were then fixed in buffered paraformaldehyde, permeabilized with Triton-X, and labelled with TUNEL reaction mixture according to the manufacturer’s instructions. Samples were analysed using a Leitz Diaplan microscope (Leica, Milan, Italy) equipped with epifluorescence. A negative control (obtained by incubating a slide with labelled solution without terminal transferase) and a positive control

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**Table 1. Oligonucleotide sequences used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
<th>Size of PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sst₁</td>
<td>5’ AGCCGTTGACTATTACGCC 3’</td>
<td>334</td>
<td>Vikic-Topic et al. (1995)</td>
</tr>
<tr>
<td>sst₂</td>
<td>5’ GCTCTCATTACCTTGATTGTTC 3’</td>
<td>461</td>
<td>Vikic-Topic et al. (1995)</td>
</tr>
<tr>
<td>sst₃</td>
<td>5’ GGTAGCTCTCTTGTTATCC 3’</td>
<td>221</td>
<td>Vikic-Topic et al. (1995)</td>
</tr>
<tr>
<td>sst₄</td>
<td>5’ TCATCTCTCTCTGCTACCTG 3’</td>
<td>247</td>
<td>Vikic-Topic et al. (1995)</td>
</tr>
<tr>
<td>SST</td>
<td>5’ CAGACTCCGTCAGTTTCCTGCA 3’</td>
<td>221</td>
<td>Kubota et al. (1994)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ GTACACCCACCATTTGGAATGTC 3’</td>
<td>264</td>
<td>Mato et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>5’ GACCCCTTCATTGACCTCAGATGTTGA 3’</td>
<td>876</td>
<td>Sinisi et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>5’ GCCAGCAGGTACGGCTGTA3’</td>
<td></td>
<td>Tso et al. (1985)</td>
</tr>
</tbody>
</table>

Reference:

- Vikic-Topic et al. (1995)
- Kubota et al. (1994)
- Mato et al. (1998)
- Sinisi et al. (1997)
- Tso et al. (1985)
(obtained by treating a slide with DNase I solution) were included in each assay run.

**cAMP production**

Confluent fibroblast monolayers in six-well plates (Costar, Milan, Italy) were starved for 24 h in medium without FCS, then transferred to medium supplemented with 1% FCS and phosphodiesterase inhibitor. The cells were treated with forskolin 10 mM, octreotide 10^{-6}, 10^{-7} and 10^{-8} M, or forskolin plus octreotide at the indicated doses. After 30 min, culture reactions were stopped by adding cold ethanol (70%). After overnight incubation in ethanol, the supernatant was collected, centrifuged and dried. The extracts were reconstituted with the appropriate buffer and processed for the assay of cAMP content using an RIA kit (Amersham).

**Electrophoresis and Western blot analysis**

Bcl-2 protein level was evaluated by Western blot analysis of protein extracts made from three different strains of fibroblast tissue. For electrophoresis and Western blot analysis, the cells were harvested after a few minutes of incubation with PBS containing 0-2 mM EDTA, centrifuged and the pellets containing 10^{-6} cells resuspended in 1:1 v/v of denaturing lysis buffer (2 x) containing 0-25 M Tris–HCl pH 6-8, 5% SDS, 8 M urea, 10 mM EDTA and 0-1 M dithiothreitol. The cell lysates were centrifuged for 10 min at maximum speed (7000 g) at room temperature to separate DNA, after which the supernatant was boiled for a few minutes before loading on gels. Protein concentrations were normalized and equal volumes of samples were loaded on the gels. Electrophoresis was performed on 12% polyacrylamide (1:40 mono-/bis-acrylamide) containing SDS, according to standard SDS-PAGE procedures. After separation on the gel, proteins were electrophoretically transferred overnight to 0-45 μM nitrocellulose sheets for Western blot analysis in transferring buffer containing 20% methanol, 10 g/l glycine, 4 g/l Tris and 0-2 g/l SDS. Nitrocellulose reactive groups were then blocked with Western blot buffer (3 g/l Na_{2}HPO_{4}, 0-3 g/l Na_{2}HPO_{4}, 12 g/l NaCl, 0-05% Nonidet P-40, 0-05% Tween-20) containing 4% non-fat dried milk (Blocker; BIORAD, Rome, Italy) and 1% BSA (pH 8-0). After 1 h of incubation with blocking solution at room temperatures, the sheets were briefly washed with Western blot buffer (pH 8-0) and incubated overnight and shaken at 4°C with primary antibodies diluted in Western blot buffer containing 1% non-fat dried milk and 0-25% BSA (pH 8-0). For Bcl-2 immunoreaction we used a monoclonal antibody (Boehringer-Mannheim, Florence, Italy) at a working concentration of 1:800. At the end of incubation, blots were washed once for 15 min and three times for 5 min with Western blot buffer (pH 8-0). The antibody reaction was revealed by incubation for 45 min at room temperature with horseradish peroxide-coupled anti-goat or anti-mouse IgG serum (Amersham), 1:10 000 diluted in Western blot buffer (pH 8-0) containing 1% non-fat dried milk and 0-25% BSA, followed by a washing cycle (as above) and using a chemiluminescent substrate (ECL; Amersham) according to the manufacturer’s instructions. Visualization was by autoradiography.

**RESULTS**

**Retroorbital fibroblasts express SST and sst genes**

Both GO and control primary cultures express the SST gene: SST gene transcripts in representative GO and control primary fibroblasts are reported in Fig. 1. One or more mRNAs of the sst_{1} family (sst_{2}, sst_{3} and sst_{4} ) were expressed in cultured cells from GO and control retroorbital tissues (Table 2). sst_{2} transcript was found in nine GO cell cultures, and was associated with sst_{3} in five, and/or with sst_{5} in eight out of the ten samples examined (see representative example in Fig. 2). sst_{2} was found in all cell cultures from control tissues, and was associated with sst_{3} in three samples (see representative example in Fig. 3). Moreover, mRNAs of the
TABLE 2. sst subtype mRNA expression in primary fibroblast cultures from retroorbital tissues

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>GO1</th>
<th>GO2</th>
<th>GO3</th>
<th>GO4</th>
<th>GO5</th>
<th>GO6</th>
<th>GO7</th>
<th>GO8</th>
<th>GO9</th>
<th>GO10</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>sst\textsubscript{1}</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sst\textsubscript{2}</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sst\textsubscript{3}</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sst\textsubscript{4}</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sst\textsubscript{5}</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

GO, Graves ophthalmopathy tissue; C, control tissue.

GO and control primary cell cultures bind \(^{[125]I-Tyr\textsuperscript{11}]SST-14\)

Three primary cultures studied displayed specific and high-affinity binding of \(^{[125]I-Tyr\textsuperscript{11}]SST-14\}. Competitive displacement by unlabelled analogues showed that the half-maximal inhibition of binding was (mean ± s.e.m.) 0·80 ± 0·37 nmol/l for SST-14 and 33·7 ± 33·1 for octreotide in GO cell cultures, and 0·9 for SST-14 and 1·5 nmol/l for octreotide in a control fibroblast culture.

In vitro effects of octreotide

GO fibroblast cultures treated with octreotide \((10^{-6} \text{ and } 10^{-7} \text{ M})\) for 72 h showed a cell growth inhibition (50 and 35% respectively) (Fig. 4), and significantly decreased \((P<0.001)\) forskolin-induced but not basal cAMP accumulation (Fig. 5). Nuclear DNA fragmentation occurred in 40 and 20% of cells exposed to \(10^{-6}\) and \(10^{-7}\) M octreotide for 72 h (Fig. 6).

Bcl-2 protein levels

We determined whether Bcl-2 proteins were present in fibroblast from GO retroorbital tissue and whether their levels changed following octreotide administration. Bcl-2 protein, detected in basal conditions, was abolished after octreotide treatment (Fig. 7).

DISCUSSION

We demonstrate that SST and its receptor genes are transcribed in primary fibroblast cultures from retroorbital tissues. We also show that retroorbital fibroblasts specifically bind SST-14, and respond in vitro to the SST analogue octreotide by inhibition of cAMP production and cell growth. The presence of functional sst\textsuperscript{s} on retroorbital tissues of patients with GO was suggested by the uptake of \(^{111}\text{In-DTPA-d-Phe}\textsuperscript{1}-octreotide into the orbits during the

sst\textsubscript{2} family were less frequently found (Table 2): sst\textsubscript{1} transcript was found in five out of the ten GO samples (see representative example in Fig. 2); sst\textsubscript{4} was absent from all samples (see representative examples in Figs 2 and 3).
active phase of the disease (Chang et al. 1991, Postena & Krenning 1994, Kahaly et al. 1995, Krassas et al. 1995, Colao et al. 1998). The retroorbital infiltrating immune cells, which harbour SST receptors, have been implicated in this uptake, but unfortunately SST receptor imaging in vivo does not distinguish the particular cells to which the labelled analogue binds (Reubi 1994). In the present study at least one of the genes coding for known SST receptor subtypes with high (sst2) or with intermediate (sst3 and sst5) affinity for the analogue octreotide appears to be expressed on GO retroorbital fibroblasts. Using competitive binding of [125I-Tyr11]SST-14, primary fibroblast cultures displayed a high affinity for SST-14 and a variable (intermediate to high) affinity for octreotide, suggesting that functional receptor proteins are present on cell membranes. Consequently, it appears that the uptake of labelled octreotide in the orbits of GO subjects in vivo may be due to specific binding to connective tissue elements and that octreotide and lanreotide may interact directly with retroorbital fibroblasts. Thus SST analogues control clinical manifestations of GO acting on immune cells infiltrating the orbits and probably on retroorbital fibroblasts.

The natural SST peptide and its stable analogues negatively regulate the growth and activity of several normal and deranged cells, including cancer cells, via specific receptor subtypes and receptor-coupled intracellular signals (Reisine & Bell 1995, Patel 1997). Octreotide, at physiological concentrations, inhibited cAMP accumulation in vitro, demonstrating that, as in other cell systems (Patel 1997), ssts in retroorbital fibroblasts are negatively coupled to adenyl-cyclase activity. In addition, octreotide significantly inhibited cell proliferation and induced signs of cell apoptosis associated with a pronounced dose-dependent inhibition of Bcl-2 protein levels, which was maximal after 72 h of treatment. Octreotide controls cell growth through subtype-specific signals: sst2 appears to be the main mediator of the tyrosine phosphatases-induced inhibition of growth factor phosphorylation; sst5 acts via the induction of retinoblastoma protein Rb and G1 cell cycle arrest; sst3 seems to mediate the cytotoxic effects of SST analogues so inducing apoptosis (Sharma et al. 1996, 1999, Patel 1997, Florio et al. 1999). The presence of both cytostatic and cytotoxic effects suggests that intracellular signal transduction pathways activated by octreotide coupling to sst2, sst3 and sst5 are functional in the retroorbital fibroblasts.

The evidence of coexpression of SST and sst genes in cultured retroorbital fibroblasts suggests that the somatostatinergic system is involved in the local control of the growth and activity of retroorbital cells in both physiological and pathological conditions. The pathogenesis of GO is unknown, but it is generally considered an extra-thyroidal manifestation of an autoimmune
process directed against an antigen common to thyroid and orbital tissue (Bahn & Heufelder 1993, Burch & Wartofsky 1993, Heufelder 1997). Experimental evidence suggests that thyrotrophin receptor (TSR), is expressed in retroorbital adipocytes, and may be one of the possible antigens stimulating the auto-antibodies implicated in proliferative changes of retroorbital tissues in GO (Bahn & Heufelder 1993). Since the SST system is involved in counteracting TSR-mediated effects on the cell cycle in thyroid cells (Medina et al. 1999), we can speculate that an autocrine loop between SST, ssts and TSR might operate in retroorbital cells and might be defective in GO.

**FIGURE 6.** TUNEL staining (A, B) and phase contrast (D, E) showing apoptotic cells in primary cultures from retroorbital fat tissue of patients affected by GO after 96 h of treatment with $10^{-7}$ M (A, D) and $10^{-6}$ M (B, E) octreotide respectively. (C, F) Untreated controls. Magnification: ×500.

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In conclusion, we found that cultured retroorbital fibroblasts expressed the SST gene together with all the sst subtypes (sst2, sst3 and sst5) that are required for the negative cell growth signal, and that bind the synthetic SST analogues currently used in clinical practice. Moreover, we demonstrate that octreotide inhibits in vitro growth and activity of retroorbital fibroblasts from GO patients, a process that could explain, in addition to the possible immune system inhibition, the reported effects of in vivo administration of SST analogues in these subjects.

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