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 (*sst*₁₋₅) 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 *sst*₂ transcript was found in nine, *sst*₃ in five and *sst*₅ in eight out of ten GO cell cultures. *Sst*₂ was detected in all six, and *sst*₃ in four out of the six control cell cultures. *Sst*₄ was absent from all samples, and *sst*₁ 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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INTRODUCTION

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

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 (*sst*_{1–5}), 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 *sst*₂, *sst*₃ and *sst*₅, which belong to the class 1 *sst* family. They do not show significant binding to *sst*₁ and *sst*₄, 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 ophthalmological 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 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 L-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 RNazol B kit (Cinna/Biotech 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 *sst*_{1–5} and SST were subjected to 25, 32, 40 and 45 amplification cycles (data not shown). The 5'-3' oligonucleotides for *sst*₁, *sst*₂, *sst*₃, *sst*₄ and *sst*₅, 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

TABLE 1. Oligonucleotide sequences used for RT-PCR

Gene	Sequence (5'–3')	Size of PCR product (bp)	Reference
<i>sst</i> ₁	5' AGCCGGTTGACTATTACGCC 3' 5' GCTCTCACTTCTACCATTGTC 3'	334	Vikic-Topic <i>et al.</i> (1995)
<i>sst</i> ₂	5' GGTGAAGTCCTCTGGAATCC 3' 5' CCATTGCCAGTAGACAGAGC 3'	461	Vikic-Topic <i>et al.</i> (1995)
<i>sst</i> ₃	5' TCATCTGCCTCTGCTACCTG 3' 5' GAGCCCAAAGAAGGCAGGCT 3'	221	Vikic-Topic <i>et al.</i> (1995)
<i>sst</i> ₄	5' CGGCAGTCTTCGTGGTCTAC 3' 5' GCATCAAGGCTGGTCACGAC 3'	247	Vikic-Topic <i>et al.</i> (1995)
<i>sst</i> ₅	5' CGTCTTCATCATCTACACGG 3' 5' GGCCAGGTTGACGATGTTGA 3'	221	Kubota <i>et al.</i> (1994)
SST	5' CAGACTCCGTCAGTTTCTGCA 3' 5' GCTAACAGGATGTGAATGTC 3'	264	Mato <i>et al.</i> (1998)
GAPDH	5' GACCCCTTCATTGACCTCAACTATG 3' 5' GTCCACCACCCTGTTGCTGTAGCC 3'	876	Sinisi <i>et al.</i> (1997) Tso <i>et al.</i> (1985)

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 [125 I-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 [125 I-Tyr¹¹]SST-14 and varying concentrations of unlabelled peptide analogues.

Cell proliferation assay

Cell proliferation was evaluated with the tetrazolium salts (MTT) method. The Cell Proliferation kit (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. Absorbancy 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

(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 ($2 \times$) 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 μ M 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_2HPO_4 , 0.3 g/l NaH_2PO_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

FIGURE 1. SST and GAPDH RT-PCR products, separated on a 1.5% agarose gel, obtained from cell cultures of retroorbital fat tissue of controls (lanes 1 and 2) and patients (lanes 3 and 4) affected by GO. SST (264 bp) and GAPDH (876 bp) mRNAs were amplified in all reactions. Lane 5 corresponds to a control RT-PCR amplification in which reverse transcriptase was excluded from the reaction. M: 100 bp DNA ladder.

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 peroxidase-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*₅) 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

	GO1	GO2	GO3	GO4	GO5	GO6	GO7	GO8	GO9	GO10	C1	C2	C3	C4	C5	C6
Cell culture																
<i>sst</i> ₁	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-
<i>sst</i> ₂	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
<i>sst</i> ₃	+	-	-	+	-	+	+	-	+	-	+	-	-	-	+	+
<i>sst</i> ₄	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
<i>sst</i> ₅	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-

GO, Graves ophthalmopathy tissue; C, control tissue.

FIGURE 2. *sst*₁₋₅ and GAPDH RT-PCR products, separated on a 1.5% agarose gel, obtained from primary cell cultures of fibroblasts from retroorbital fat tissue of a patient affected by GO. Transcripts *sst*₁ (334 bp), *sst*₂ (461 bp), *sst*₃ (221 bp) and *sst*₅ (223 bp) are shown. Note that the GAPDH transcript is consistently amplified (876 bp). M: 100 bp DNA ladder.

FIGURE 3. *sst*_{2,3} and GAPDH RT-PCR products, separated on a 1.5% agarose gel, obtained from cell cultures of retroorbital fat tissue of a control patient. *sst*₂ (461 bp) and *sst*₃ (221 bp) mRNAs are the only transcripts present; GAPDH (876 bp) is amplified in all reactions. M: 100 bp DNA ladder.

*sst*₂ family were less frequently found (Table 2): *sst*₁ transcript was found in five out of the ten GO samples (see representative example in Fig. 2); *sst*₄ was absent from all samples (see representative examples in Figs 2 and 3).

GO and control primary cell cultures bind [¹²⁵I-Tyr¹¹]SST-14

Three primary cultures studied displayed specific and high-affinity binding of [¹²⁵I-Tyr¹¹]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 and 10⁻⁷ 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 *ssts* on retroorbital tissues of patients with GO was suggested by the uptake of [¹¹¹In-DTPA-D-Phe¹-octreotide into the orbits during the

FIGURE 4. Octreotide-induced growth inhibition of cell cultures from retroorbital fat tissue of patients affected by GO. The cells were seeded at 2000 cells per well and treated with 10^{-6} M and 10^{-7} M octreotide (OCT) for 72 h. The results were expressed as the A_{550} of MTT-derived formazan developed by untreated control (C) and treated cells. Each point represents the mean $\pm$ s.e. of three separate experiments (* $P < 0.05$; ** $P < 0.0005$).

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

FIGURE 5. Octreotide-induced inhibition of forskolin (FK)-stimulated cAMP accumulation in cell cultures from retroorbital fat tissue of patients affected by GO. The cells were treated with FK 10 µM with or without octreotide (OCT) 10^{-6} M and 10^{-7} M for 30 min. Each column represents the mean $\pm$ s.e. of three separate experiments (* $P < 0.001$ versus FK-stimulated cAMP accumulation).

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 [125 I-Tyr 11]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 adenylyl-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

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: $\times 500$.

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 7. Western blot analysis of Bcl-2 protein expression in cell lysate of fibroblasts from GO retroorbital tissue before (lane 1) and after 72 h (lane 2) of 10^{-6} M octreotide treatment.

In conclusion, we found that cultured retroorbital fibroblasts expressed the SST gene together with all the *sst* subtypes (*sst*₂, *sst*₃ and *sst*₅) 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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