Octreotide promotes apoptosis in human somatotroph tumor cells by activating somatostatin receptor type 2

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Abstract

Somatostatin analogs currently used in the treatment of acromegaly and other neuroendocrine tumors inhibit hormone secretion and cell proliferation by binding to somatostatin receptor type (SST) 2 and 5. The antiproliferative pathways coupled to these receptors have been only partially characterized. The aim of this study was to evaluate the effect of octreotide and super selective SST2 (BIM23120) and SST5 (BIM23206) analogs on apoptotic activity and apoptotic gene expression in human somatotroph tumor cells. Eight somatotroph tumors expressing similar levels of SST2 and SST5 evaluated by real-time PCR and western blot analyses were included in the study. In cultured cells obtained from these tumors, octreotide induced a dose-dependent increase of caspase-3 activity ($160 \pm 20\%$ vs basal at 10 nM) and cleaved cytokeratin 18 levels ($172 \pm 25\%$ vs basal) at concentrations higher than 0.1 nM. This effect was due to SST2 activation since BIM23120 elicited comparable responses, while BIM23206 was ineffective. BIM23120-stimulated apoptosis was dependent on phosphatases, since it was abrogated by the inhibitor orthovanadate, and independent from the induction of apoptosis-related genes, such as p53, p63, p73, Bcl-2, Bax, BID, BIK, TNFSF8, and FADD. In somatotroph tumors, both BIM23120 and BIM2306 caused growth arrest as indicated by the increase in p27 and decrease in cyclin D1 expression. In conclusion, the present study showed that octreotide-induced apoptosis in human somatotroph tumor cells by activating SST2. This effect, together with the cytostatic action exerted by both SST2 and SST5 analogs, might account for the tumor shrinkage observed in acromegalic patients treated with long-acting somatostatin analogs.

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Introduction

Somatostatin is a widely distributed polypeptide that predominantly exerts inhibitory effects on hormone secretion and cell proliferation by interacting with five different subtypes of receptors, named somatostatin receptor type (SST) 1 through 5. The binding of somatostatin to the receptor activates a number of G protein-linked transduction pathways, including adenylyl cyclase, calcium channels, phosphotyrosine

phosphatase, and mitogen-activated protein kinase, largely depending on the SST subtype and the cell system considered (Hofland *et al.* 1999, Benali *et al.* 2000). Due to the physiological actions of somatostatin, long acting analogs that bind with high affinity SST2 and, to a less extent, SST5, such as octreotide and lanreotide, have been developed for the medical treatment of neuroendocrine tumors, in particular acromegaly (Hofland *et al.* 1999, Patel 1999, Lamberts

et al. 2002, Weckbecker *et al.* 2003, Oberg *et al.* 2004, Bevan 2005, Freda *et al.* 2005).

The mechanisms mediating the antiproliferative action of somatostatin and the receptor subtypes involved in this process have been only partially characterized. In particular, studies on cell lines expressing SST1, SST2, SST4, and SST5 showed that somatostatin caused cell cycle arrest by inducing retinoblastoma tumor suppressor protein (Rb) and cyclin-dependent kinase inhibitors, such as p27, p21, and p16 (Srikant 1995, Pages *et al.* 1999, Sharma *et al.* 1999). In contrast, until recently, SST3 was considered the receptor subtype mainly involved in the induction of cell death, although in some cell systems SST2 was shown to trigger both cytostatic and cytotoxic responses (Srikant 1995, Sharma *et al.* 1996, Gruszka *et al.* 2001). Consistent with these data, we recently reported the ability of octreotide to induce apoptosis in human somatotroph tumors, this response being associated with a reduced expression of seladin-1, a putative antiapoptotic gene (Luciani *et al.* 2005).

The aim of this study was to identify the receptor subtype involved in the proapoptotic action of octreotide by evaluating the effect of superselective somatostatin analogs on caspase activity and apoptosis-related gene expression in human somatotroph tumors.

Materials and methods

Pituitary tissue samples and cell cultures

The study included somatotroph tumors surgically removed from acromegalic patients, who did not receive medical treatment with somatostatin analogs before surgery ($n=8$). The removed tissues were in part quickly frozen for subsequent molecular studies and in part placed in sterile medium for cell culture, after the exclusion of blood cell contamination, as previously described (Lania *et al.* 2004). Local ethical approval was obtained for the study.

SST2 and SST5 mRNA and protein quantification

To quantify SST2 and SST5 mRNA levels, total RNA was isolated from tumor tissues, reverse transcribed (primers and amplification conditions available on request) and evaluated by real-time reverse transcription-polymerase chain reaction (RT-PCR) based on TaqMan methodology, as previously reported (Luciani *et al.* 2005). Total proteins were extracted from the same tissues, homogenized with PBS and quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Twenty micrograms of protein were resolved on

10% SDS-PAGE gel and transferred onto nitrocellulose membrane. Polyclonal antibodies specific for SSTR2 and SSTR5 were obtained from PRIM Laboratories (Milan, Italy) and used according to the manufacturer indications. The visualization and reading of the resulting bands were analyzed, as previously described (Lania *et al.* 2004). Experiments were repeated at least twice. To detect the specificity of the reaction, the antiserum was preincubated with the peptide against which the antibody was raised. As control, preparations of CHO-K1 cells transfected with human SST5 were positive for SST5 and negative for SST2 at immunoblotting (Ballarè *et al.* 2001). The expression of glyceraldehyde-3-phosphatedehydrogenase (GAPDH) by a mouse monoclonal anti-GAPDH antibody (Ambion Ltd, Huntingdon, Cambridgeshire, UK) was used as an internal control for protein loading.

SST analogs

The following SST analogs were used: octreotide with a specificity (IC_{50} , nM) of 0.56 for SST2 and 7 for SST5; BIM23120 with a specificity of 0.34 for SST2, and 213 for SST5 and BIM23206 with a specificity of 166 for SST2 and 2.4 for SST5 (Ren *et al.* 2003). The superselective analogs were kindly provided by Biomeasure Incorporated/IPSEN (Milford, MA, USA).

Caspase-3 activity

Caspase-3 enzymatic activity was measured using Apo-ONE Homogenous Caspase-3 Assay (Promega). Briefly, tumoral cells were seeded in 96-well plates (50 000 cells/well) and treated with or without octreotide, BIM23120, BIM23206 (at 0.1, 1, 10, and 100 nM) for 6 h at 37 °C, in Dulbecco's modified essential medium (DMEM, Sigma Chemical) supplemented with 10% FCS. The timing of treatment was determined on the basis of preliminary experiments carried out at different time intervals showing the maximal stimulation at 6 h. Experiments were repeated at least twice and each determination was done in quintuple.

Cleaved cytokeratin (CK) 18 determination

Cleaved CK18 levels were measured using M30-Apoptosense ELISA (Peviva-Alexis, Bromma, Sweden). Briefly, 100 000 tumoral cells were seeded in six-well plates and incubated in the presence or absence of either octreotide or BIM23120 or BIM23206 for 16 h at 37 °C and cell extracts were used for the assay, as previously described (Luciani *et al.* 2005). Experiments were repeated at least twice and each determination was done in triplicate.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis

Apoptosis was also evaluated by TUNEL analysis, using a commercially available detection kit (APO-DIRECT, Phoenix Flow Systems, Inc., San Diego, CA, USA), as previously described (Luciani *et al.* 2005). Briefly, cells obtained from two growth hormone-(GH) secreting adenomas were seeded in coverslips and incubated in the presence or absence of somatostatin analogs (10 nM) for 16 h at 37 °C. The cells were fixed in 1% paraformaldehyde in PBS, pH 7.4, treated with 70% ice ethanol for 30 min at –20 °C, exposed for 60 min at 37 °C to the DNA labeling solution and observed using a transmission fluorescence microscope (Leitz, Type 307-148002, Wetzlar, Germany) equipped with an E4 filter (Leica, Milan, Italy). Images were captured by a Canon digital camera using Remote Capture software (provided by Canon, Machida, Japan) and edited by Adobe photoshop version 5.0 (Adobe Systems Inc.).

Cyclin D1 and p27 expression

After 24 h of serum starvation, tumoral cells were incubated with BIM23120 or BIM23206 (10 nM) for 8 h at 37 °C. The determination of cyclin D1 was performed after immunoprecipitation of cell lysates with specific monoclonal antibodies (Novocastra, Newcastle, UK) and western blotting, as previously reported (Corbetta *et al.* 2005). The determination of p27 was performed after immunoprecipitation of cell lysates with specific polyclonal antibody (Upstate Europe, Dundee, UK) according to manufacturer instructions. Experiments were repeated at least twice. The expression of GAPDH by a mouse monoclonal anti-GAPDH antibody (Ambion Ltd) was used as an internal control for protein loading.

Real-time PCR for apoptosis-related genes

For the evaluation of the expression of nine apoptosis-related genes (p53, p63, p73, Bcl-2, Bax, BID, BIK, TNFSF8, and FADD), total RNA obtained from cultured cells from three tumors after short-(30 min) and long-term (16 h) incubation with or without octreotide or BIM23120, was isolated, reverse transcribed, and evaluated by real-time RT-PCR, as previously described (Nosotti *et al.* 2005). Briefly, for the quantification of the three internal controls and the nine apoptosis-related genes we used a ready-to-use assay (Assay-on-Demand Gene Expression Products) purchased from Applied Biosystems. It consists of a 20× mix of unlabeled PCR primers and TaqMan

MGB probe (6-FAM at 5' end and a no fluorescent quencher at 3' end). The assay identification numbers of selected genes were: Hs00153340_m1 (TP53), Hs00186613_m1 (TP73L, p63), Hs00232088_m1 (TP73), Hs00153350_m1 (Bcl-2), Hs00180269_m1 (Bax), Hs00609630_m1 (BID), Hs00609635_m1 (BIK), Hs00174286_m1 (TNFSF8), and Hs00538709_m1 (FADD).

Materials

Octreotide (SMS 201-995) was provided by Novartis Pharma. DMEM, sodium orthovanadate, fetal calf serum (FCS) and okadaic acid were obtained from Sigma Chemical.

Statistical analysis

Results are expressed as the mean $\pm$ s.d. A paired or unpaired two-tailed Student's *t*-test was used to detect the significance between two series of data. $P < 0.05$ was accepted as statistically significant.

Results

SSTR2 and SSTR5 expression

The tumors included in the study showed clearly detectable levels of SST2 and SST5 mRNA and proteins. In particular, no significant difference between SST2 and SST5 mRNA levels was observed (Fig. 1A), despite certain variability among the different tumors (Fig. 1A). Moreover, receptor proteins were detected by specific antisera directed against SST2 and SST5 in all tumors (Fig. 1B), without striking differences among the individual tumors and clear correlations with mRNA levels.

Effect of selective SST agonists on apoptosis and cyclin D1 expression

In cultured cells obtained from all tumors, octreotide induced a dose-dependent increase in caspase-3 activity, that was detected at concentrations higher than 0.1 nM and maximal at 10 nM ($160 \pm 20\%$ vs basal, $P < 0.05$) (Fig. 2). The limited amount of tissue available did not allow an accurate evaluation of the concentrations giving the half-maximal effect (ED50) that, however, resulted within the 0.5–2 nM range. The selective SST2 analog BIM23120 induced an increase in caspase-3 activity comparable with that caused by octreotide ($185 \pm 45\%$ vs basal, at 10 nM, $P < 0.05$) in term of efficacy and potency, while the selective SST5 analog BIM23206 was ineffective (Fig. 2). The effects

Figure 1 SST2 and SST5 expression in human somatotroph tumors. (A) SST2 and SST5 mRNA levels were detected in eight adenomatous tissue samples obtained by surgery, using real-time PCR technique. (B) Representative immunoblotting performed in four somatotroph tumors with antibodies raised against SST2 and SST5. C-, tumor tissue subjected to antiserum preincubated with 0.5 μg/μl peptide at 4 °C for 12 h.

of octreotide and BIM23120 on caspase activity were similar to that induced by the aspecific proapoptotic agent okadaic acid ($205 \pm 25\%$ vs basal, $P < 0.05$; Fig. 2). Similar results were obtained by evaluating the effects of different somatostatin analogs on cleaved CK18 levels, a product of caspase-3 activation ($172 \pm 25\%$ vs basal, $P < 0.05$; Fig. 3). The stimulatory effects on both caspase-3 activity and CK18 levels were prevented by treating cultured cells with the phosphotyrosine phosphatase inhibitor orthovanadate (25 μM; Figs 2 and 3). In these tumors, apoptosis was also assessed by TUNEL analysis, which is able to reveal the total number of apoptotic cells, independent of the activated pathways. As shown in Fig. 4, the number of apoptotic nuclei was markedly increased after BIM23120 exposure (10 nM). No significant correlation between SST2 and SST5 mRNA and protein expression and caspase-3 activity and CK18 levels stimulation was observed.

Finally, in cultured cells obtained from three GH-secreting adenomas, both BIM23120 and BIM23206 (10 nM) induced a reduction of about 50% in cyclin D1 expression (Fig. 5A) and an increase of about 200% in P27 expression (Fig. 5B).

Effect of SST analogs on apoptosis-related genes

Incubation of tumoral cells from three somatotroph tumors with octreotide (10 nM) and BIM23120 (10 nM) for 3 or 16 h did not induce any significant changes in p53, p63, p73, Bcl-2, Bax, BID, BIK, TNFSF8, and FADD mRNA levels as demonstrated by real-time quantitative RT-PCR (data not shown).

Discussion

This study confirmed that octreotide was able to induce apoptosis in human somatotroph tumor cells and provided novel evidence for the selective involvement of SST2 receptor in this process. Previous studies indicated that somatostatin and its analogs exert direct antiproliferative effects with cytostatic (growth arrest) and cytotoxic (apoptosis) consequences. The cytostatic effects of somatostatin have been documented in multiple cell types, including GH3 and AtT20 pituitary tumor cell lines (Cheung & Boyages 1995, Srikant 1995). Studies performed in cells stably expressing the cDNA encoding the human SST2 and SST5 sequences strongly supported the involvement of these receptor subtypes in the cytostatic action of somatostatin. In particular, in these systems somatostatin caused cell cycle arrest in G1, due to upregulation of Rb and cyclin-dependent kinase inhibitors, such as p27, p21, and p16 (Pages *et al.* 1999, Sharma *et al.* 1999). The cytostatic action of somatostatin was confirmed by the present study that showed a significant increase in cyclin-dependent kinase inhibitors p27 expression together with a significant reduction of cyclin D1, a cell cycle progression protein, by both SST2 and SST5 selective analogs in somatotroph tumors.

Until recently, SST3 was considered the only receptor subtype able to trigger apoptotic signals (Sharma *et al.* 1996). Subsequently, it has been demonstrated that also SST2 may initiate apoptotic pathways in cell lines expressing human SST2, including AtT20 pituitary cells (Srikant 1995, Gruszka *et al.* 2001). Consistent with recent data from our laboratory (Luciani *et al.* 2005), octreotide was able to increase the enzymatic activity of caspase-3, a major protease mediator of the extrinsic and intrinsic apoptotic pathway, in all somatotroph tumors investigated. Moreover, the present study clearly identified SST2 as the receptor subtype involved in the proapoptotic action triggered by octreotide in these tumors. In fact, although the superselective analog of SST3 was not available for a direct receptor activation, the involvement of SST3 in

Figure 2 Effects of somatostatin analogs on apoptosis in human somatotroph tumors: (A) octreotide induced a dose-dependent increase in caspase-3 activity, that was maximal at 10 nM and similar to the increase induced by the aspecific proapoptotic agent okadaic acid (100 nM). The selective SST2 analog BIM23120 caused a dose-dependent increase in caspase-3 activity that was prevented by the phosphatase inhibitor orthovanadate (25 μ M). (B) Conversely, the selective SST5 analog BIM23206 did not significantly affect apoptosis in any GH-omas. Experiments were repeated at least twice and each determination was done in quintuple. Data represent the percent increase (mean $\pm$ s.d.) of both caspase-3 activity over basal values, arbitrarily defined as 100%, in eight GH-omas * P <0.05 vs basal; † P <0.05 vs BIM23120.

octreotide-induced apoptosis seems unlikely, taking into account the low affinity of octreotide for this receptor together with the low expression of SST3 found in somatotroph tumors, in comparison with SST2 and SST5 (Ren *et al.* 2003, Luciani *et al.* 2005). Moreover, octreotide and BIM23120 concentrations effective in activating the apoptotic process resulted to be in the dose range reported to inhibit *in vitro* GH release (Ren *et al.* 2003).

The mechanisms involved in growth arrest and apoptosis induced by somatostatin are only partially elucidated. However, accumulating evidence indicate that cell cycle arrest is largely mediated by SST2-induced activation of SHP-1 and SHP-2, two members of the Src homology 2 (SH2) containing tyrosine phosphatase family (Pages *et al.* 1999, Ferjoux *et al.* 2003). In addition to inducing cell arrest, tyrosine

Figure 3 Effect of somatostatin analogs on cleaved CK18, a product of caspase-3 activation. Both octreotide (10 nM) and the selective SST2 agonist BIM23120 (10 nM) induced a significant increase in cleaved CK18 levels that was prevented by the phosphatase inhibitor orthovanadate (25 μ M). No effect was induced by the selective SST5 agonist (BIM23206, 10 nM). Data represent the percent increase (mean $\pm$ s.d.) of cleaved CK18 levels over basal values, arbitrarily defined as 100%, in eight GH-omas * P <0.05 vs basal; † P <0.05 vs BIM23120.

Figure 4 Determination of apoptotic nuclei by TUNEL analysis in cells from somatotroph tumors both in basal conditions (A) and upon BIM23120 treatment (B). Green fluorescence (right) and bright images (left) from the same field are shown. A few apoptotic nuclei were observed in basal conditions (A), whereas nuclear fluorescence markedly increased after BIM23120 exposure, as shown in B. Magnification 100 $\times$.

phosphatases have been demonstrated to represent essential components of somatostatin-mediated cytotoxic signaling that causes intracellular acidification and apoptosis in MCF-7 human breast cancer cells (Thangaraju *et al.* 1999). Consistent with the view that SST2 signals by recruiting and activating phosphotyrosine phosphatases, initiation of apoptotic pathway by octreotide and BIM23120 in tumoral somatotroph cells required the activation of these enzymes.

We also investigated the induction of apoptosis-related genes by SST2 activation in human somatotroph tumor cells. On the basis of intrinsic cell death pathway previously reported for SST3 that involved p53 and Bax induction (Sharma *et al.* 1996), the expression of genes belonging to p53 family (p53, p63, and p73) and to two subfamilies of proapoptotic Bcl-2 family (Bax and BH3-only) was investigated. In addition, extrinsic apoptotic pathway was partially

studied through FADD and TNFSF8 expression. Incubation with octreotide and BIM23120, both after short and long-term, did not induce significant modifications on gene expression. Accordingly, it has been reported that SST2 may induce death of HL-60 cells, a cell line with a null p53 phenotype due to p53 gene deletion (Teijeiro *et al.* 2002). Moreover, as observed HL-60 cells (Teijeiro *et al.* 2002), SST2 activation did not modify the expression of other candidate genes, such as p73, that can induce apoptosis even in the absence of p53 (Zhu *et al.* 2001). In this respect, it has been proposed that cytosolic accumulation of p53 may activate Bax and trigger mitochondrial membrane permeabilization and apoptosis by a transcription-independent mechanism (Chipuk *et al.* 2004). Further studies are needed to ascertain the cell death pathways underlying the apoptotic effect induced by SST2 analog in somatotroph tumor cells,

Figure 5 Representative immunoblotting showing the effect of SST5 and SST2 activation on cyclin D1 expression in cultured cells obtained from three GH-omas. Both SST2 (BIM23120) and SST5 (BIM23206) selective analogs reduced cyclin D1 (A) and increased expression of cyclin-dependent kinase inhibitors p27 (B). GAPDH protein expression has been used as an internal control.

that resulted to be different from those previously reported for SST3 (Sharma *et al.* 1999).

Apoptosis is a physiological process of programmed cell death triggered by a number of stimuli, including hormones, DNA damage, and anticancer drugs. Moreover, apoptosis in response to antineoplastic agents correlates with tumor shrinkage. The presence of morphological apoptotic features, such as cell shrinkage, nuclear condensation, membrane blebbing and DNA fragmentation, in surgical specimens obtained from acromegalic patients chronically treated with long acting somatostatin analogs has been reported by some authors and negated by others (Losa *et al.* 2001, Wasko *et al.* 2003). Despite these discrepant data, it is tempting to speculate that the *in vitro* cytotoxic effects induced by SST2 activation in somatotroph tumor cells may be involved in the tumor shrinkage observed in a consistent proportion of acromegalic patients treated with somatostatin analogs (Bevan 2005, Freda *et al.* 2005). Admittedly, large series of tumors, including those resulted to be resistant to octreotide *in vivo* and removed after an appropriate washout period, would be required in order to assess the possible correlation between *in vitro* proapoptotic action of sst2 analog and *in vivo* antitumoral action of octreotide.

In conclusion, the present study showed that octreotide-induced apoptosis in human somatotroph

tumor cells by activating SST2. This effect, together with the cytostatic action exerted by both SST2 and SST5 analogs, might account for the antineoplastic effect of long-acting somatostatin analogs in acromegaly.

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