Somatostatin and dopamine receptor expression in lung carcinoma cells and effects of chimeric somatostatin-dopamine molecules on cell proliferation

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Submitted 10 May 2005; accepted in final form 21 July 2005

Am J Physiol Endocrinol Metab 289: E1044–E1050, 2005. First published July 26, 2005; doi:10.1152/ajpendo.00209.2005.—To study somatostatin/dopamine (SS/D) synergy in a human cell system constitutively expressing SS and D receptors (SSR and DR, respectively), we characterized the expression of SSR and DR subtypes in the non-small-cell lung cancer line Calu-6, and then we evaluated the effect on cell proliferation of SS/D chimeric molecules (BIM-23A387 and BIM-23A370), which bind with high affinity both ssst2 and D2R, and compared the results with those obtained by using SS-14 and subtype-selective SS analogs (SSA) and D agonists (DA). Because Calu-6 cells produce insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) peptides, which play a role in the autocrine/paracrine control of cell growth, we also investigated the effects of chimeric compounds on secretion and expression of IGF system components. Relative high levels of sst2 and the long isoform of the D2R were detected by real-time RT-PCR and Western blot in Calu-6, together with ssst5 and to a lesser extent ssst3 and D2R. BIM-23A387 and BIM-23A370 significantly inhibited growth of Calu-6, whereas IGF-IGFBP secretion or expression was unaffected, suggesting a direct inhibitory effect. The inhibition of cell growth, measured by both [3H]thyidine incorporation and cell count, was significantly lower when individual SSA and DA control peptides or subtype-specific SSA and DA were tested. BIM-23A370 was more potent than BIM-23A387 (P < 0.001). These findings show that SS/D chimeras can inhibit Calu-6 proliferation in an IGF-independent manner and suggest that this enhanced potency might be because of the induction of SSR/DR dimerization. The Calu-6 cell line, constitutively expressing SSR and DR, provides a suitable model to elucidate the mechanism of action of SSA and DA on regulation of cell growth and to characterize the interaction between SSR and DR.

somatostatin receptors; dopamine receptors; receptor dimerization; growth factors; lung carcinoma

Somatostatin (SS) plays an important regulatory role in controlling the function of various cells, including inhibition of hormone release, immunomodulation, neurotransmission, and inhibition of cell proliferation and angiogenesis (8, 2). Most of the effects of SS and of its currently available analogs (SSA), octreotide, lanreotide, and vapreotide, are mediated via a family of high-affinity G protein-coupled membrane receptors (SSR) with five known subtypes, termed ssst1–5 (15). All the genes for SSR are intronless except the ssst2, which contains a cryptic intron sequence and encodes for two receptor proteins, ssst2A and ssst2B that originate from alternative splicing. SSR are expressed in a tissue- and subtype-selective manner in both normal and neoplastic cells, and the majority of SS target tissues express multiple SSR subtypes at the same time (21, 29). Similarly, five different dopamine receptors (D1–5R), also belonging to the G protein-coupled family of receptors, have been cloned and characterized so far (14). Alternative splicing of the gene encoding the D2R leads to the short (D2Rs) or the long (D2Rl) isoforms. SSA and new potent dopamine agonists (DA), such as cabergoline and quinagolide, are routinely used, alone or in combination, for medical treatment of pituitary adenomas. These compounds not only inhibit hormone secretion but can also reduce tumor mass (27). Moreover, recent data suggest that membrane SSR and DR may interact to form heterodimers with enhanced functional activity (23). However, receptor dimerization has thus far only been demonstrated in transfected cell systems and in rat neuronal cells (1). It is of great interest to investigate whether such a phenomenon occurs in human cells constitutively coexpressing SSR and DR. Recently, SSA/DA chimeric molecules were created that contain structural elements of both SS and dopamine (D). These hybrid molecules retain potent, selective agonist activity on both the ssst2 and the D2R, and their activities are currently being investigated in different cell systems (20, 26).

Human small cell lung cancer (SCLC) and non-SCLC (NSCLC) may express SSR to a greater extent than normal tissue, as has been demonstrated both in vivo and in vitro (11, 16, 18). Interestingly, dopamine receptors (DR) are expressed in human lung tumor as well, and DA has been shown to inhibit the growth of human SCLC xenografts in athymic nude mice in a dose-dependent manner (7, 28). Calu-6 is an established human NSCLC cell line producing a large amount of insulin-like growth factor (IGF)-II, a lesser amount of IGF-I, and low amounts of IGF-binding proteins (IGFBPs), which play a critical role in the autocrine/paracrine control of cell growth. Among different cell lines available in our laboratory, we finally selected these human cells on the basis of their peculiar pattern of neuropeptide receptor expression (see below). In this study, we used Calu-6 cells, already characterized in our laboratory for IGF autocrine stimulating pathways (9, 10),

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13), to investigate: 1) the expression of SSR and DR subtypes; 2) the effects on cell proliferation of two new SS/DA chimeric molecules (BIM-23A370 and BIM-23A387) with the ability to bind both sst2 and D2-R with high affinity compared with the effect of experimental and clinically available SSA and DA agonists; and 3) the effects of chimeric molecules on the production and expression of IGF system components. Briefly, we attempted to ascertain whether SS/DA chimeric compounds, either directly or via modification of the IGF system, influence the differentiation and proliferation of this cell line and whether these cells, constitutively expressing SSR and DR, may represent a suitable model to further investigate neuropeptide receptor cross-talk and regulation.

MATERIALS AND METHODS

Products. BIM compounds, including lanreotide, were provided by Biomeasure (Milford, MA). BIM-23A370 and BIM-23A387 are chimeric molecules that combine structural elements of both SS and D and retain affinity for both the sst2 and D2-R. BIM-23023 is an sst2-preferential compound, and BIM-53097 is a D2-R-preferential compound that served as control peptides for chimeras (28). BIM-23206 is an sst5-preferential compound, whereas BIM-23244 is a bispecific sst2/sst5-preferential ligand (27). The IC50 values for BIM compounds are reported in Table 1. The DA agonist cabergoline was kindly provided by Pharmacia, while SS-14 was purchased from Bachem (Hanover, Germany).

Cell cultures. Cells were grown in MEM supplemented with 10% FCS, 1% nonessential amino acids, 20 mg/dl gentamycin, 200 mM glucose, and 1 mM sodium pyruvate until subconfluent. Different cultures were settled for functional studies in MEM serum-free medium supplemented with 0.1% BSA. Calu-6 cells (1 × 105) were seeded in 0.2 ml culture medium in 96-well plates for proliferation studies. To assess the effects of test substances on IGF-II and IGFBP secretion, cells (1 × 105) were seeded in 24-well plates.

Cell proliferation. SS/DA chimeric compounds (BIM-23A387 and BIM-23A370), control peptides (BIM-23023 and BIM-53097), BIM-23206, BIM-23244, lanreotide, and cabergoline were added to the cultures at concentrations ranging from 10⁻² to 10⁻⁷ M. After 24 h, proliferation was measured by cell counting and by measuring thymidine incorporation after adding 1 µCi [³H]thymidine (Amersham) during the last 6 h of incubation. Cell viability was constantly tested before and after the functional studies and was satisfactory. [³H]Thymidine incorporation was determined after harvesting cells on glass fibers and liquid scintillation counting on a scintillation counter. Results are calculated as average counts per minute of quadruplicate wells ± SE.

IGF-II and IGFBP secretion. After 18 h in serum-free medium, Calu-6 cells were incubated in fresh medium with or without SS/DA chimeric compounds and control peptides at a concentration of 10⁻⁸ M. After 24 h, conditioned medium was collected and dialyzed against 1 M acetic acid. IGFs were separated from IGFIBPs by gel filtration on a FPLC Superdex-75 column equilibrated with 0.1 M acetic acid-0.15 M NaCl. IGF content was evaluated in the eluted fractions in the molecular weight range of the free peptide, whereas the IGFFB content was evaluated in the fractions eluted in the molecular mass range of >20 kDa by appropriate immunoassay, as already reported (6).

RT-PCR studies. RT-PCR for SSR and DR was performed as previously described (4). Briefly, poly(A)⁺ mRNA was isolated using Dynabeads oligo(dT)₂₅ (Dyman, Oslo, Norway) from cell pellets containing 1–2 × 10⁶ cells/sample. cDNA was synthesized using the poly(A)⁺ mRNA captured on the Dynabeads oligo(dT)₂₅ as solid-phase and first-strand primer. One-tenth of the cDNA was used for each amplification by PCR using primer sets specific for human sst₁₋₅, D₁₋₅-R, and hypoxantine guanine phosphoribosyltransferase (HPRT). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly(A)⁺ mRNA preparation (since the SSR subtype genes are intronless), the cDNA reactions were also performed without RT and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as a positive control for the quality of the cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the SSR receptor subtypes, 0.001–0.1 mg human genomic DNA, representing ~300–30,000 copies of sst template, was amplified in parallel with the cDNA samples. As positive controls for the PCR reactions of the DR subtypes and HPRT, 0.01 µg human brain cDNA was amplified in parallel with the cDNA samples of Calu-6 cells. For quantitative RT-PCR, the 5’-exonuclease (TaqMan) assay, which produces a direct proportional readout for the progression of PCR, was used. Amplification of cDNA derived from 50–150 ng total RNA, obtained from Calu-6 cells, was performed in a 25-µl reaction volume with 300 nm of each primer, 200 nm of the probe, and 12.5 µl MasterMix (PE Applied Biosystems, Paris, France). The probe comprised 20–30 nucleotides with 5’-end substitution with a fluorophore and a quencher substitution at the 3’-end. The synthetic sst and D2-R cDNA primers used in the PCR were 19- or 20-mers as follows: sst₂₅_A (GenBank accession no. M81830), sense (10–29), antisense (109–91), and probe (58–32); D₂₋₅-R (Gen-Bank accession no. AF050737 coding sequence), sense (1062–1083), antisense (1177–1156), and probe (1088–1115). The annealing-extension temperatures were 59°C for sst₂ and 60°C for D₂₋₅. Forty cycles of two-step PCR-annealing extension, at specified temperatures for 60 s, and denaturation at 95°C for 15 s were performed on an ABI Prism 7700 sequence detection apparatus (PE Applied Biosystems). The sst and D₂₋₅-R mRNA levels were normalized to the β-glucuronidase (β-Gus) mRNA levels obtained in the same reaction. The β-Gus primers and probe were purchased from PE Applied Biosystems. For each measurement, three independent RT-PCR analyses were performed. To produce standard curves for sst₂₋₅, sst₅, sst₂, D₂₋₅-R mRNA, and β-Gus mRNA, cDNA constructs were produced for each parameter. Using the specific

Table 1. Human dopamine and sst binding affinities of chimeric compounds, dopamine, and somatostatin analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>D₂-R</th>
<th>sst₁</th>
<th>sst₂</th>
<th>sst₃</th>
<th>sst₄</th>
<th>sst₅</th>
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</thead>
<tbody>
<tr>
<td>BIM-23A370</td>
<td>26.9</td>
<td>826</td>
<td>0.01</td>
<td>ND</td>
<td>61</td>
<td>31</td>
</tr>
<tr>
<td>BIM-23A387</td>
<td>25.5</td>
<td>293</td>
<td>0.1</td>
<td>77.4</td>
<td>ND</td>
<td>23</td>
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<tr>
<td>BIM-23023</td>
<td>&gt;1,000</td>
<td>6,616</td>
<td>0.42</td>
<td>86.9</td>
<td>2,700</td>
<td>4.18</td>
</tr>
<tr>
<td>BIM-53097</td>
<td>22.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BIM-23206</td>
<td>ND</td>
<td>1,152</td>
<td>166</td>
<td>1,000</td>
<td>1,618</td>
<td>2.4</td>
</tr>
<tr>
<td>BIM-23244</td>
<td>ND</td>
<td>1,020</td>
<td>0.29</td>
<td>133</td>
<td>&gt;1,000</td>
<td>0.67</td>
</tr>
<tr>
<td>Lanreotide</td>
<td>ND</td>
<td>2,129</td>
<td>0.75</td>
<td>98</td>
<td>1,826</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Data from radioligand assays using membranes from transfected CHO-K1 cells expressing the human dopamine type 2 receptor (D₂-R) or the human somatostatin (sst) subtypes. sst₁₋₅, 5 subtypes of somatostatin receptors. Values are from Biomeasure (Taylor and Culler, personal communication). ND not done.
fluorogenic probes for each gene under the experimental conditions defined above, we obtained a linear relationship between the cDNA concentration and the threshold cycle of fluorescent signal for sst2A, sst3, sst5, D2R, and β-Gus from 50 to 5,000,000 copies of cDNA target. For each unknown sample, we determined the threshold cycle values for sst2A, sst5, and D2R, and the results were expressed as copies of sst2, sst3, sst5, or D2R copies of β-Gus.

The IGF system components, RNA extraction and RT-PCR were performed only for IGF-II and IGFBP-2, the two components mainly expressed by this cell line, as already described (17).

Immunoblotting. Membrane receptors were obtained from Calu-6 cells by solubilization in lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml bacitracin, and 4 mg/ml N,N,N-triacetylchitotriose) for 1 h at 4°C and subsequent centrifugation for 1 h at 4°C and subsequent centrifugation for 1 h at 100,000 g. Glycosylated proteins were then immobilized by recycling the solubilized membranes overnight at 4°C through a 0.5-ml wheat-germ agglutinin (Vector Laboratories, Burlingame, CA) column that had been equilibrated with lysis buffer. The column was washed and eluted with lysis buffer containing 3 mM N,N,N-triacetylchitotriose (Sigma-Aldrich, St. Louis, MO). The protein content of the eluted protein was assessed by Bradford assay. Glycosylated proteins (10 μg) was denatured and fractionated on 12.5% SDS-PAGE and then transferred electrophoretically to Hybond C-extra nitrocellulose membranes. After transfer, nonspecific binding sites were blocked by treating membranes with Tris-buffered saline-Tween (TBS-T: 0.02 M Tris base, 0.137 M NaCl, and 0.5% Tween 20; pH 7.6 with 1 M HCl) containing 5% nonfat dried milk for 1 h at 22°C on a rotating shaker. After five washes with TBS-T, membranes were incubated for 1 h at 4°C with a 1:500 dilution of sst1, sst2A, sst3, sst5 (Biotrend, Cologne, Germany), and D2R (Chemicon, Temecula, CA) polyclonal antibodies in TBS-T containing 1% BSA. Membranes were washed five times with TBS-T and then incubated for 1 h at 22°C with 1:2,000 dilution of horseradish peroxidase-linked anti-rabbit IgG, washed as before and immersed for 0.5–1 min in the chemiluminescence detection solution. Subsequently, membranes were exposed for ~0.5 min to generate immunoblots.

Statistical analysis. Statistical analysis was performed by parametric ANOVA for unpaired samples and area under the curve computation (AUC) followed by ANOVA and t-test. P < 0.05 was considered significant. Results are expressed as means ± SE. To minimize variation among different experiments, the results are expressed as relative variation from the untreated control value (Δvalue).

RESULTS

Somatostatin and dopamine receptor subtype expression. The expression of sst2, sst3, sst5 and of D2R, as well as D3R, was detected by both RT-PCR and Western blot in Calu-6 NSCLC. The RT-PCR and Western blot results are shown in

Fig. 2. Western blot analyses of somatostatin receptor subtypes sst2, sst3, and sst5 and dopamine receptor subtype D3R in human Calu-6 NSCLC cells.
Figs. 1 and 2. Real-time RT-PCR revealed a lower expression of sst$_2$, sst$_3$, sst$_5$, and D$_2$R mRNA than nine pituitary tumors from acromegalic patients known to express both SSR and DR and to be responsive to SSA and DA (28). The expression of D$_2$R (1.7 D$_2$R/β-Gus copy mRNA) was higher than that of all SSR subtypes. Among SSR subtypes, the expression of sst$_5$ (0.5 sst$_5$/β-Gus) was relatively higher than that of sst$_2$ (0.008 sst$_2$/β-Gus), whereas the expression of sst$_3$ was very low (0.001 sst$_3$/β-Gus).

Functional studies. These studies were performed to test the effects of the chimeric compounds, the control peptides for the chimeras, SSR subtype-preferential compounds, as well as SS-14, cabergoline, and lanreotide on Calu-6 cell proliferation and production of IGF/IGFBP peptides.

Cell proliferation. All tested substances significantly inhibited cell proliferation, as measured by cell counting (data not shown) and by $[^3]$H thymidine incorporation, but to different extents ($P < 0.0001$). The analogs do not have a significant effect at all concentrations tested, but the curves were different and the concentrations significantly affected the result. Results from the dose-response experiments are illustrated in Fig. 3. Both chimeras, BIM-23A370 and BIM-23A387, resulted in significantly more potent inhibition of cell proliferation compared with all the other compounds, tested alone or in combination, including SS-14, the sst$_2$-preferential compound BIM-23023, and the D$_2$R-preferential compound BIM-53097 (controls for the chimeras). Moreover, the BIM-23A370 was significantly more potent than the other chimera, BIM-23A387 (Fig. 4). SS-14 was significantly more effective in inhibiting $[^3]$H thymidine incorporation compared with lanreotide, the sst$_2$- and sst$_5$-preferential compounds BIM-23023 and BIM-23206, alone or combined, cabergoline, and the combination of the sst$_2$- and D$_2$R-preferential compounds BIM-23023 and BIM-53097, whereas it was not significantly different from the bispecific sst$_2$/sst$_5$ analog BIM-23244 and the combination of cabergoline plus lanreotide. These results, obtained after the...
calculation of the AUCs, are summarized and expressed in Fig. 4.

**IGF system secretion and expression.** The BIM-23A370 chimera and the DA and SS controls BIM-23023 and BIM-53097 were ineffective in modulating either the expression or the secretion of IGF and IGFBP peptides in cultured Calu-6 cells (data not shown). Figure 5 shows the lack of effect of chimera and control peptides on IGF-II and IGFBP-2 mRNA expression, the main IGF and IGFBP peptides produced by Calu-6 cells.

**DISCUSSION**

The coadministration of SSA and DA is considered more effective than treatment with either drug alone in suppressing growth hormone (GH) secretion and normalizing circulating IGF-I levels in a minority of acromegalic patients (3, 5, 10, 12). These are mainly patients bearing mixed pituitary tumors that cosecrete both GH and prolactin (PRL). These tumors are known to express a higher number of DR on tumor cells than on “pure” GH-secreting adenomas. However, a recent study demonstrated the lack of additive effect of an sst2-prefering agonist (BIM-23023) and a D2R-prefering agonist (BIM-53097) in suppressing GH and PRL release in cultured human pituitary adenoma cells from patients that were both fully and partially responsive to SSA (26). Conversely, an enhanced potency in suppressing hormone secretion, compared with either single or combined administration of SSA and DA, was observed in the same cases when a new chimeric molecule (BIM-23A387) was used (26). The authors hypothesized that the increased potency of this new compound, which retains somatostatinergic and dopaminergic activities in the same molecule, might be due to ligand-induced SSR and DR dimerization, resulting in the formation of a novel receptor with distinct functional properties (26). Receptor dimerization is well known to occur for G protein-coupled membrane receptors, including SSR and DR (15, 19, 22, 23, 24). Members of both SSR and DR superfamilies may interact within the plasma membrane, forming both homo- and heterodimers. Heterodimerization of sst3 and D2R was demonstrated in CHO-K1 cells, in which both receptor subtypes were cotransfected (23). The sst3 and D2R dimerization in the CHO-K1 cells resulted in a new dimeric entity with increased ligand binding affinity and enhanced functional activity (23). In contrast, again in transfected human HEK-293 cells, sst2 and sst3 heterodimerization resulted in the inactivation of sst3 (19). However, apart from preliminary data in rat neuronal cells (1), receptor dimerization has so far been observed only in transfected cell systems, and
the significance and the properties of the dimeric form of these receptors has never been clarified.

In the present study, we investigated a human tumor cell line constitutively expressing both SSR and DR. In particular, we demonstrated the presence of sst2 and sst5, together with D2R and to a lesser extent sst3 and D4R, at both the nucleotide and protein levels in the Calu-6 NSCLC line. We therefore focused our attention on the two specific receptor subtypes already established to be involved in receptor dimerization, namely sst2 and D2R. We studied the effects of different compounds with specific binding profiles, including chimeric molecules that selectively bind both sst2 and D2R, on cell proliferation and on the IGF system autocrine pathway.

In dose-response studies, both chimeric compounds, BIM-23A370 and BIM-23A387, were found to be significantly more potent in inhibiting cell proliferation than specific sst2-, sst3-, and D2R-preferential ligands, including lanreotide and cabergoline, on cell growth is in line with the very low expression of their related SSR and DR subtypes, whereas the chimeric molecules strongly affect cell proliferation. Indeed, the inhibitory activity of one chimera (BIM-23A370) was higher than that of the other and even more pronounced when compared with SS-14 and with the currently available SSA and DA lanreotide and cabergoline, tested alone and in combination.

Because Calu-6 cell growth is regulated by an autocrine pathway involving specific components of the IGF system (9, 13), in particular IGF-II, we evaluated the effects of the more potent chimera (BIM-23A370) and its control peptides (the sst2-prefering analog BIM-23023 and the D2R-prefering analog BIM-53097) on the secretion and synthesis of IGFs and IGFBPs; there was no effect whatever. The results of these experiments exclude an IGF-mediated inhibition of cell growth for SSA, DA, and the SS/DA chimeric compounds. On the basis of their affinity profile, we currently have no explanation for the enhanced potency of the chimera BIM-23A370 compared with the other chimera BIM-23A387. However, conformational differences, stability, and altered ability to induce receptor activation and/or cooperation cannot be ruled out. Excluding both chimeras, the next most potent compound was SS-14, and this evidence might be explained by the SS-14-induced recruitment of additional SSR subtypes, namely sst3 and sst5, which are also coexpressed in these cells, or, even more interesting, by a ligand-induced SSR dimerization. The evidence that the bispecific sst2/sst3 SSA BIM-23244 displayed a higher potency in inhibiting cell proliferation than lanreotide, as well as the sst2- and sst5-prefering analogs, but not of SS-14, strongly supports this hypothesis. On the other hand, this bispecific SSA has already been demonstrated to achieve better control of GH hypersecretion than single subtype-specific analogs in a number of pituitary tumors heterogeneously expressing sst2 and sst5 receptor subtypes (25). SSR/DR dimerization might be the basis for the observed enhanced potency of the chimeric compounds in inhibiting in vitro Calu-6 cell proliferation. Although further studies are needed to directly demonstrate that these chimeric molecules might act via the induction of receptor dimerization, they represent a novel tool for investigating SSR and DR properties and unraveling new potential signal transduction pathways in cells coexpressing SSR and DR.

In conclusion, we showed for the first time that the new SS/DA chimeric molecules BIM-23A387 and BIM-23A370, which simultaneously bind both sst2 and D2R, may directly inhibit in vitro tumor cell growth to a greater extent than SSA and DA tested either alone or in combination. This enhanced potency might be related to the capacity of these new compounds to induce SSR/DR dimerization. Finally, Calu-6 NSCLC displays a specific SSR and DR profile and represents an interesting model for the study of neuropeptide receptor interaction and signal transduction pathways in human cancer.

GRANTS

This work was supported by research grants from Fondo per gli Investimenti della Ricerca di Base (FIRB RBAU019TMF_001) and Ministero dell’Università e della Ricerca Scientifica (2002067251-001).

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