Somatostatin analogues inhibit growth of pancreatic cancer by stimulating tyrosine phosphatase
(oncogenes/tyrosine kinase/epidermal growth factor/receptors)

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ABSTRACT Several analogues of somatostatin were examined in the Mia PaCa-2 human pancreatic cancer cell line for their ability to promote tyrosine phosphatase activity affecting the receptors for the epidermal growth factor. The inhibition of growth of the Mia PaCa-2 cells in culture was also evaluated to determine the mechanism of action of somatostatin analogues and their relative effectiveness in inhibiting cancer growth. Of the analogues tested D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH2 (RC-160) caused the greatest stimulation of tyrosine phosphatase activity. Analogue D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH2 (RC-121) had less effect but was more potent than somatostatin-14. Analogue D-Phe-Cys-Phe-d-Trp-Lys-Thr-Cys-Thr(ol) (SMS 201-995) produced no significant dephosphorylation. The analogues displayed the same order of activity in assays on growth inhibition of Mia PaCa-2 cells in cultures. Analogue (SMS-201-995) caused virtually no tyrosine phosphatase stimulation or growth inhibition in this cancer cell line, although it possesses a much higher antisecretory activity than somatostatin-14 in normal tissues. These observations indicate that somatostatin and some of its analogues can act as growth inhibitors in cancer cells through the activation of tyrosine phosphatase. These data reinforce the view that somatostatin analogue RC-160 and related compounds could be used for treatment of pancreatic cancer.

Somatostatin is a hormone widely distributed in the body, which, in addition to endocrine actions, can also have exocrine and paracrine effects (1). Somatostatin can exert inhibitory actions on various tissues and affect many cellular functions (1–3). Receptors for somatostatin that mediate these actions have been found in normal tissues and in tumors (3–5). Two well studied cellular processes affected by somatostatin are secretion and growth (1–5). The processes of secretion by many different cells and tissues can be inhibited by somatostatin (2, 3). Among the effects induced by somatostatin is the inhibition of pancreatic exocrine secretion of protein and bicarbonate and of endocrine events, including the reduction in the release of insulin and glucagon (2, 4). Somatostatin-14 and its analogues have been used clinically to treat various tumors and other conditions (5–7).

Inhibition of tumor growth by somatostatin could occur through the suppression of secretion of various hormones and/or by direct effects on the neoplasm itself. For pancreatic cancers, somatostatin inhibits the release of cholecystokinin, gastrin, and secretin (1–4), which have trophic effects on the growth of the pancreas and, possibly, on pancreatic tumors (5, 8). The Mia PaCa-2 human undifferentiated pancreatic cancer cell line, developed by Yunis (9), has been shown to have somatostatin receptors (10). Somatostatin inhibited epidural growth factor (EGF)-stimulated growth of Mia PaCa-2 line in cell cultures (11). This demonstrated that somatostatin could inhibit pancreatic cancer growth not only indirectly through the modulation of circulating hormone levels but also through direct effects.

The somatostatin receptor–somatostatin complex in Mia PaCa-2 cells could act as a specific tyrosine phosphatase (10), and this activity could be related to the growth-inhibiting effects obtained (11). The relationship between tyrosine phosphorylation and growth stimulation was suggested by the finding of parallel stimulation of both growth and tyrosine kinase activity by the EGF–EGF receptor complex (12) and the erbB oncogene product (13, 14). The finding of an independent enzyme acting as a tyrosine phosphatase and a growth inhibitor supports the hypothesis of a functional relationship between phosphorylation and growth (13, 15–17), although some observations indicate that phosphorylation and growth may vary independently in some cells (18, 19). Additional evidence could help to elucidate these phenomena.

An implication of this relationship between tyrosine phosphatase and growth inhibition would be that somatostatin could serve as a therapeutic agent in the treatment of pancreatic cancer and other tumors. However, it is well established that therapeutic use of somatostatin is impractical because of its short half-life in circulation (5) and broad spectrum of activities (1–4). To circumvent these problems, analogues of somatostatin with enhanced selectivity and greater stability have been developed (1–7, 20, 21). One of these is the octapeptide analogue D-Phe-Cys-Phe-d-Trp-Lys-Thr-Cys-Thr(ol) (SMS-201-995) of Bauer et al. (20). This analogue has been demonstrated to be an effective antisecretory agent with proven clinical effectiveness in acromegaly, endocrine tumors of the gut and pancreas, and other conditions (7). Various octapeptide analogues, including D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH2 (RC-160) and D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH2 (RC-121) developed by Schally and coworkers (3, 5, 21), have also been found to be highly active, long-acting, and endowed with significant antitumor activity after in vivo administration (5, 22, 23). In the studies reported herein, we examined the ability of these somatostatin analogues to directly inhibit the growth of Mia PaCa-2 cells in cultures and to stimulate the tyrosine phosphatase activity of somatostatin receptors in this cell line.

MATERIALS AND METHODS

Peptides. The tetradecapeptide somatostatin-14 (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) was made in our laboratory or obtained from Novabiochem, Laufelfingen, Switzerland. Analogues D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH2 (RC-121) and D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr(ol) are widely distributed in the body, which, in addition to endocrine actions, can also have exocrine and paracrine effects (1). Somatostatin can exert inhibitory actions on various tissues and affect many cellular functions (1–3). Receptors for somatostatin that mediate these actions have been found in normal tissues and in tumors (3–5). Two well studied cellular processes affected by somatostatin are secretion and growth (1–5). The processes of secretion

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Abbreviations: EGF, epidermal growth factor; FBS, fetal bovine serum; RC-160, D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH2; RC-121, D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr(ol); SMS-201-995, D-Phe-Cys-Phe-d-Trp-Lys-Cys-Thr(ol).
d-Trp-Lys-Val-Cys-Trp-NH₂ (RC-160) were synthesized in our laboratory by solid-phase methods and repurified by HPLC (21). Both analogues were about 100 times more potent than somatostatin on inhibition of growth hormone release (21). These analogues also possessed antitumor activity in vivo on prostate and pancreatic cancers (22, 23) and caused a powerful inhibition of exocrine and endocrine pancreatic secretion (4). Analogue d-Phe-Cys-Phe-d-Trp-Lys-Thr-Cys-Thr-(ol) (7, 20) containing a C-terminal amino alcohol was obtained from Sandoz Pharmaceutical. All the three cyclic octapeptide analogs share some common amino acid sequences (3–5, 21).

**Cell Growth.** Mia PaCa-2 cell line was obtained from American Type Culture Collection. The three analogues were tested at various doses in the Mia PaCa-2 cells. Each experiment was conducted as follows. The cells were grown in 75-cm² flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS) at 37°C in 5% CO₂/95% air until they reached 75% confluency. After incubation, the cells were harvested with 4 ml of 10% wt/vol trypsin solution. The cells were then placed into 200 ml of fresh medium with FBS. While the solution was continuously stirred, 4 ml aliquots of the cell solution were transferred to 48 Petri dishes. Aliquots of the cell solution were transferred at random to assure equal distribution. The plates were incubated for 24 hr to allow for complete reattachment of the cells to the plates. The medium was then changed to DMEM without FBS, to unmask any endogenous growth factors that may exist in the FBS, and incubated for 24 hr. After this period, each plate received analogue and 10 nM EGF and was then incubated again for 24 hr. The cells were harvested by trypsinization and counted using a Coulter counter to determine the growth rates of the cells. Each of these experiments was counted as one independent trial.

**Phosphatase Assay.** Phosphatase assays were performed as described (10). Mia PaCa-2 cell membranes from cells grown to confluence, scraped mechanically from the Petri dish, and isolated by homogenization and by differential centrifugation (12) were prelabeled with [γ-³²P]ATP (20 μM) in the presence of 10 μM EGF at 0°C for 60 min in 20 mM Hepes buffer (pH 7.5) containing 2.0 mM MnCl₂. Cell membranes were incubated in the presence of an excess of unlabeled ATP (0.8 mM) at 0°C for 60 min in the absence or somatostatin or one of the three analogues at concentrations from 10⁻¹¹ M to 10⁻⁶ M (10). EGF receptor was purified by SDS gradient gel electrophoresis, and radioactivity in the 170-kDa band was measured (10). The effects of the analogues were compared to the dephosphorylation produced by 1 nM of the synthetic somatostatin-14.

**RESULTS**

When EGF receptor was prelabeled with 120 pmol of ³²P per mg of protein, the incubation in the absence of somatostatin-14 or its analogues produced no measurable dephosphorylation. Incubation with somatostatin-14 produced 38% dephosphorylation, as compared to 2.5% for the analogue SMS-201-995, and 46% and 100%, respectively, for the analogues RC-121 and RC-160 (Table 1). A dose–response curve suggested that the maximal rate of phosphatase activity produced by the peptides differed for each analogue, with the RC-160 being the most active, RC-121 significantly less effective, and somatostatin-14 still less active (Fig. 1). No significant dephosphorylation was produced at any concentration of SMS-201-995, up to the 1 mM concentration tested. RC-121 and somatostatin-14 had similar half-maximal effective doses in the range of 1 nM. Because RC-160 produced 100% dephosphorylation, we were not able to accurately compute its half-maximal effective dose, but it did not appear to be greatly different from that of RC-121 or somatostatin-14.

**Table 1.** Stimulation of phosphatase by somatostatin analogues

<table>
<thead>
<tr>
<th>Peptide added</th>
<th>³²P released, pmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>SMS-201-995</td>
<td>3.0</td>
</tr>
<tr>
<td>Somatostatin-14</td>
<td>45.6</td>
</tr>
<tr>
<td>RC-121</td>
<td>55.0</td>
</tr>
<tr>
<td>RC-160</td>
<td>120.0</td>
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Release of ³²P from prelabeled Mia PaCa-2 membranes without (control) or with somatostatin or its analogues at 1 nM.

The growth rates of the Mia PaCa-2 cells were 100% per day in the presence of 10 nM EGF corresponding to a 24-hr doubling time. The maximal growth inhibition was produced by analogue RC-160. This inhibition was greater than that obtained with RC-121, which in turn caused much higher suppression than SMS-201-995. Analogue RC-160 caused a significant inhibition (P < 0.01) at all doses tested, with the maximal response being seen with the lowest dose, 1 pM (Fig. 2). RC-160 produced a final cell count corresponding to 62% of control values, obtained with cells grown in the presence of EGF, but in the absence of analogues. Since at the time of addition of the analogue, we start with 50% of the cell number found after 24 hr of incubation with EGF alone, 62% of control value signifies only 24% growth or 76% less growth than found in the absence of analogue RC-160. With the analogue RC-121, there was a significant inhibition (P < 0.05) at 1 pM and 1 nM, but the decrease was not as great as that obtained with RC-160. Analogue RC-160 produced an average inhibition of 76% at the four doses, whereas RC-121 produced 53% suppression in cell number. The analogue SMS-201-995 failed to show any significant inhibition at the doses tested.

**DISCUSSION**

Our results demonstrate that somatostatin octapeptide analogues RC-160 and RC-121 inhibit the growth of MIA PaCa-2 pancreatic cancer cells and stimulate tyrosine phosphatase activity by a direct action. These data are consistent with the findings (5, 10) that some human and experimental pancreatic cancer cells release somatostatin-14.

![Fig. 1. Effect of somatostatin-14 (SS-14) and its analogues, RC-160, RC-121, and SMS-201-995, on dephosphorylation of the EGF receptor derived from human pancreatic Mia PaCa-2 cancer cells. A starting sample with the 170-kDa membrane protein (i.e., EGF receptor) labeled with 120 pmol of ³²P per mg of protein was incubated in the absence (control, □) or presence of SS-14 (○) or its analogues, SMS-201-995 (●), RC-121 (△), and RC-160 (▲), for 60 min at 0°C. SMS-201-995 had no effect on promoting phosphatase activity, RC-121 and SS-14 were effective, and RC-160 was most active.](image-url)
cancers have somatostatin receptors. It has also been demonstrated that the growth of pancreatic cancers in rats and hamsters is inhibited in vivo by administration of somatostatin analogues, including RC-160 (5, 23, 24). Our work indicates that somatostatin and its analogues can directly inhibit pancreatic cancer growth, in addition to exerting suppressive effects on the release or action of cholecystokinin, gastrin, and secretin, which could also indirectly result in retardation of cancer growth.

A similar spectrum of activity among somatostatin analogues on growth inhibition and stimulation of tyrosine phosphatase of EGF receptors supports the hypothesis that these two phenomena are causally related. The relationship between phosphorylation of tyrosine residues of proteins and growth control has been suggested by Ushiro and Cohen (25). Additional evidence for this relationship comes from the similar tyrosine kinase activity of erbB and EGF receptors (14, 26). Bishop (15) linked oncogene misinformation to sequentially linked transducer systems of tyrosine kinase and serine kinase and provided a global model for growth regulation. This sequential application of a tyrosine kinase system that subsequently activates a serine kinase system has been explored in the transduction system of insulin by Yu and Czech (16). They demonstrated the sequential autophosphorylation of the β subunit of insulin on a tyrosine residue, followed by tyrosine kinase phosphorylation of a putative acceptor protein, which then phosphorylates a serine kinase on a tyrosine residue and activates it. This cascade of events permits the amplification of the growth signal and also allows for the multiple inputs that may together affect and control cellular growth. Our evidence that the analogues of somatostatin influence both growth inhibition and tyrosine phosphatase in a parallel manner according to their potency, complements the observations of Yu and Czech (16) that some modulators of insulin-activated phosphotyrosine kinase also affect the phosphorylation of the acceptor protein and the ensuing cascade of steps results in growth regulation.

Although the evidence linking enhanced tyrosine kinase activity to growth promotion seems strong, there is also evidence to the contrary. Schreiber et al. (18) uncoupled EGF-stimulated phosphokinase activity and DNA synthesis in the A-431 cell line by cleaving EGF with cyanogen bromide. The cleaved EGF was capable of stimulating autophosphorylation of EGF receptor but not the DNA synthesis. They concluded from this observation either that phosphorylation is not the transducing signal for EGF or that it is not the sole signal. Buss et al. (19) found that in variants of A-431 cells, a parallelism existed between the number of EGF binding sites, protein kinase stimulating ability of EGF, and its capacity to inhibit, instead of stimulating, the growth of these cells. These observations cast some doubt on whether the phosphorylation at a tyrosine residue is the signal for growth stimulation. One possible explanation for these apparent discrepancies stems from the fact that in both of these studies the A-431 cells were used. Others have noted that A-431 cells are unusual in that EGF may not be a classical growth stimulator for these cells but instead might not affect or might even inhibit growth (16).

Our evidence that somatostatin and its analogues can activate an independent specific tyrosine phosphatase (10) and inhibit growth (11) through a mechanism independent of the EGF receptor–tyrosine kinase system supports the hypothesis that these phenomena are functionally linked. Our findings that somatostatin analogues affect these two processes to a similar extent further support the hypotheses of a functional relationship. Other studies suggesting the existence of two EGF receptors (27), or of two forms of the EGF receptor (17), may provide another explanation for the discrepancies noted by some investigators between the processes of phosphorylation and growth (18, 19). The complexity of the message transduction process itself may also help explain these discrepancies. Yu and Czech (16) envision the multistep process of insulin message transduction as an initial autophosphorylation of the β subunit of the insulin receptor, followed by its activation of tyrosine kinase phosphorylation of the P160 protein, and finally by the phosphorylation and activation of a serine kinase by this phosphorylated P160 protein. This model is analogous to that shown in Figs. 3 and 4 for phosphotyrosine kinase activities. EGF receptor and erbB. Two separate tyrosine phosphorylation events occur in this system, (i) the autophosphorylation of EGF receptor and erbB and (ii) the phosphorylation of an acceptor protein, for instance P160 in the insulin system. It is suggested in Fig. 4 that the phosphorylation of the tyrosine kinase (either the EGF receptor or the erbB) provides a phosphate to be transferred to the acceptor protein. This is conjectural, because it is possible that the phosphorylation event on the receptor (or oncogene) merely activates the protein. However, it is an attractive hypothesis to equate these phosphorylation reactions with the reduction of the energy of activation of the enzymatic phosphorylation reaction. Because multiple steps involving phosphorylation at tyrosine residues are implicated in the cascade of growth regulation and because somatostatin can promote dephosphorylation at more than one of these steps (10), somatostatin may influence a variety of cells in different ways depending on which phosphorylated protein is most affected in each cell (5, 17, 27).

Even though the analogue SMS 201-995 has been proven to be a powerful inhibitor of pancreatic secretions (4), in our studies in the in vitro model, it lacked the ability to directly inhibit pancreatic cancer cell growth. Our data also indicate that the somatostatin receptor in Mia PaCa-2 pancreatic cancer cells involved in stimulating dephosphorylation of endogenous phosphorylated EGF receptor recognizes the endogenous tetradecapeptide somatostatin and two of its octadecapeptide analogues, RC-160 and RC-121, but not the
Growth Control Through Phosphorylation

![Diagram showing the process of growth control through phosphorylation](image)

Fig. 3. Hypothetical scheme of the role of tyrosine phosphatase in growth regulation in normal and cancerous cells. The scheme shows known steps of tyrosine phosphorylation in the growth regulation pathway, stimulated by EGF [with normal EGF receptor (EGFR) or excessive quantities of EGF receptor] or activated constitutively with the oncogene erbB. Somatostatin (SS) has been shown to be active in dephosphorylation of EGF receptor and, therefore, in inhibiting growth in step 1 in this pathway. Somatostatin may also be involved in other steps identified as “SS?”.

 analogue SMS-201-995. The two analogues RC-121 and RC-160 activated the receptor more powerfully than somatostatin, with the RC-160 being the most potent. The fact that the analogue SMS-201-995 is effective in inhibiting endocrine and exocrine secretion of the normal pancreas, but ineffective in stimulating the dephosphorylation of EGF receptor and inhibiting cell growth in our pancreatic model, implies that the cancer cells may have dissimilar receptors from the normal pancreas or that a different receptor from that which regulates EGF receptor dephosphorylation could govern secretory responses. This suggests the possibility that distinct somatostatin receptors are expressed in the normal versus cancerous tissues in analogy to the different EGF receptors (17, 27). The differences between somatostatin receptors in pancreatic cancer cells and in normal tissue were not identified in our studies. However, the possibility of the existence of two classes of receptors for the same ligand is intriguing. Our findings may support the hypothesis that EGF and its related onconecines could act as growth promoters directly through their effect on tyrosine kinase activation. Our studies imply that different types of somatostatin receptors might exist for the regulation of normal and cancerous tissues or for inhibiting secretory function and growth.

Finally, our observations also suggest that analogue RC-160 would appear to be the most potent, among those examined, for treatment of pancreatic cancer. Our results support the findings of previous in vivo studies (5, 23, 24) that analogues of somatostatin should be considered for the development of additional therapy for pancreatic cancer.

Fig. 4. A scheme of the molecular event producing phosphorylation signal. Several phosphorylation steps exist, including one on the tyrosine kinase enzyme itself (EGF receptor or erbB), and another on a mobile acceptor. The scheme implies that the kinase transfers the phosphate from itself to the acceptor, but this is not proven. Any event that reduces the phosphorylation signal, by phosphatase inactivation of the kinase or by dephosphorylation of the soluble phosphorylated tyrosine protein, would slow the growth.

The phosphatase experiments were performed by Dr. Marion Hierowski, who is now deceased. Though Dr. Hierowski did not have the opportunity to contribute to the preparation of the manuscript, he provided an inspiration and impetus for the accomplishment of this work. This manuscript is, therefore, dedicated to Marion Hierowski for his intellectual, spiritual, and personal contribution to this work in particular and science in general. This work was supported by the Smokeless Tobacco Research Council, the Margaret Duffy and Robert Cameron Troup Memorial Fund, and the Buffalo General Hospital (to C.L.) and Grant CA 40077 from the National Institutes of Health and the Medical Research Service of the Veterans Administration (A.V.S.).