Antiproliferative Effects of the Somatostatin Analogue Octreotide (SMS 201–995) on ZR-75–1 Human Breast Cancer Cells in Vivo and in Vitro

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ABSTRACT

The somatostatin analogue octreotide (SMS 201–995) inhibits secretion and growth of certain tumor cells, and current efforts are directed toward the elucidation of its mode of antiproliferative action. In this study, the effect of octreotide on the growth of ZR-75–1 human breast cancer cells has been characterized in immunodeficient nude mice and in cell culture. These results have been related to the expression of somatostatin receptors in vivo and in vitro. Continuous infusion of 10 μg/kg/h of octreotide yielded plasma levels of 5.7 ng/ml and elicited highly significant growth inhibitory effects on solid ZR-75–1 breast tumors in nude mice. After 2 and 4 weeks of treatment, tumor volumes in the octreotide group were 39.1 and 36.7% of those of control animals treated with vehicle, respectively. Autoradiographic studies demonstrated that 8 of 12 ZR-75–1 tumors studied were somatostatin receptor positive. When ZR-75–1 tumor cells were exposed in vitro to nanomolar concentrations of octreotide, a dose-dependent inhibition of cell growth was observed in the presence of 5% fetal calf serum or under serum-free conditions using epidermal growth factor, insulin-like growth factor type I, or insulin as growth stimulus. In parallel receptor-binding experiments, ZR-75–1 cells were shown to express specific high-affinity somatostatin receptors (Kd value = 0.9 nM, Bmax = 6000 sites/cell). From these experiments, we conclude that octreotide is a powerful inhibitor of ZR-75–1 tumor cell growth in nude mice and in culture. This inhibitory action of octreotide and the presence of somatostatin receptors on ZR-75–1 tumor cells in vitro and in vivo suggest a direct, somatostatin receptor-mediated effect of octreotide.

INTRODUCTION

Octreotide (SMS 201–995, Sandostatin) is a synthetic peptide derivative of natural somatostatin with markedly increased metabolic stability which results in an increased plasma half-life of >1 h. The analogue is highly effective in inhibiting hormone secretion and is used for the treatment of various endocrine and malignant disorders including acromegaly (1) and gastroenteropancreatic tumors (2). The inhibitory effects of octreotide are mediated by specific high-affinity somatostatin receptors, the function of which is being intensely investigated (3). Somatostatin exerts a direct antiproliferative effect on HeLa and gerbil fibroma cells in culture (4), and also, octreotide can inhibit the growth of tumor cells in culture and tumors in animal models (5–14). Clinical studies revealed occasional tumor shrinkage in gastroenteropancreatic tumor patients treated with octreotide (2). The expression of specific somatostatin receptors has been demonstrated in several types of human tumors including neuroendocrine, central nervous system, lung, and breast malignancies. At least 40% of human breast tumors were shown by autoradiography to be somatostatin receptor positive (15). The latter finding, along with the well-known dependence of mammary tumor growth on hormones and growth factors, makes breast tumors an interesting target for exploring the role of octreotide in cancer treatment.

In the present study, a well-characterized human breast cancer cell line (ZR-75–1) was used to investigate the growth inhibitory effect of octreotide on solid tumors growing in nude mice and on cells in culture. Simultaneously, the receptor status for somatostatin, IGF-I,2 and EGF was determined. The presence of somatostatin receptors on such tumors may indicate their sensitivity to octreotide treatment.

MATERIALS AND METHODS

Materials. EGF and insulin were purchased from Sigma (Zurich, Switzerland). IGF-1 was supplied by KabiGen (Stockholm, Sweden). Labeled growth factors were purchased from Amersham (Zurich, Switzerland). Tyr-3-octreotide was iodinated in-house using the Bolton-Hunter method. Media and fetal calf serum were supplied by Flow Labs (Irvine, Scotland). Cell culture plates were supplied by Falcon (Dietikon, Switzerland). Minipumps were purchased from Alzet/Ch. River (Cleon, France).

Cell Culture. The human breast cancer cell lines ZR-75–1 (estrogen dependent) and MDA-MB-231 (estrogen independent) were obtained from American Type Culture Collection and regularly checked for the absence of Mycoplasma using bisbenzimide staining and the GenProbe hybridization assay (San Diego, CA). Cultures were propagated in RPMI 1640 medium (ZR-75–1) or improved modified Eagle’s medium (MDA 231) supplemented with 5% FCS at 5% CO2.

Proliferation Experiments. To investigate tumor cell proliferation in the presence of drug, stock cultures at approximately 80% confluence were harvested by trypsinization and seeded in 24-well plates at a density of 3–5 x 104 cells/well. Assays were usually set up in quadruplicate. After a 1- to 2-day attachment period, the medium was replaced by a fresh one containing 5% FCS and octreotide. The number of cells was determined with a Coulter Counter type ZM. In further experiments, serum-free conditions were established subsequent to cell attachment with the respective medium containing 0.1% BSA. Following a 1- to 3-day incubation in the absence of serum, the medium was replaced by a fresh one containing 0.1% BSA, growth factors, and octreotide at concentrations as indicated in the respective figures and legends.

Somatostatin-binding Assay. Cells were grown in 12-well plates for at least 5 days before binding experiments were performed. Cells were incubated with the radioligand 125I-Tyr3-octreotide (90 000 cpm/well) for 30 min at 37°C in HEPES buffer (10 mM, pH 7.5) containing 2 μg/ml bacitracin, 0.5% BSA, and 5 mM MgCl2. Incubation was stopped by repeated washing of the cells with cold HEPES buffer (pH 7.5) and incubating with 10% sodium dodecyl sulfate to detach cells. Nonspecific binding of the radioligand was determined in the presence of 100 nM unlabeled octreotide. Cell-bound radioactivity was counted in an LKB gamma counter.

Determination of Octreotide in Plasma. Plasma levels of octreotide in nude mice bearing ZR-75–1 tumors and osmotic minipumps (octreotide infusion rate, 10 μg/kg/h) were determined according to standard procedures (1). Briefly, mice were anesthetized with isoflurane (Abbott, Cham, Switzerland), and blood was collected from the retroorbital plexus into EDTA-coated tubes on day 7 of the 14-day infusion period. The radioimmunoassay was carried out by incubating the plasma with rabbit anti-octreotide serum (1:150,000) and 125I-Tyr3-octreotide (10,000 cpm) for 24 h at 4°C. Dextran-coated charcoal was used to...
separate bound from free tracer. Radioactivity was determined in a LKB gamma counter.

 Autoradiographic Studies. Previously described autoradiographic procedures were used (16). Briefly, frozen tissue samples were sectioned at −20°C using a microtome cryostat and mounted on gelatin-coated glass slides. Somatostatin receptors were labeled with the radioiodinated 125I-Tyr3-octreotide (16). Autoradiograms were generated by exposing the labeled tumor sections to tritium-sensitive Ultrasfilm (LKB, Broma, Sweden) for 10 days.

EGF-binding Assay. ZR-75-1 cells were grown in 24-well plates for 6–8 days until they reached confluence. The cells were washed with buffer (Hanks' solution plus 2 mg/ml BSA) at 0°C and kept on ice for 30 min. Following the addition of increasing concentrations of 125I-EGF (Amersham; specific radioactivity, 0.17 μCi/ml), cells were incubated for 1 h, then washed with buffer, and trypsinized for determination of bound radioactivity in a gamma counter. Nonspecific binding of 125I-EGF was determined in the presence of 100 nM unlabeled EGF.

IGF-I-binding Assay. Membrane preparations of confluent ZR-75-1 cells were obtained by differential centrifugation of cell homogenates in HEPES buffer (20 mM, pH 7.4) containing 250 mM sucrose, 0.1 mM EDTA, 1 mM benzamidine, 50 μg/ml soybean trypsin inhibitor, 1 μM leupeptin, 0.1 μM aprotinin, and 1 μM pepstatin A. The membranes were incubated with 125I-labeled recombinant human IGF-I (50,000 cpm; Amersham) for 1 h at 37°C in 30 mM HEPES containing 1 mg/ml BSA, 1 mM benzamidine, and 1 μg/ml leupeptin. Nonspecific binding of 125I-IGF-I was determined in the presence of 100 nM unlabeled IGF-I. Incubations were terminated by the addition of 4 ml ice cold tris-buffered saline (10 mM, pH 7.4) and rapid filtration using Millipore HVLP 02500 filter discs.

Nude Mouse Experiments. Female nude mice (nu/nu, IFFA C, BALB/c) weighing 20–23 g were kept in Macrolon cages (type III, 16 x 22 x 11 cm; Eh Emmendinger, Germany) placed in ventilated closed cabinets (IFFA Credo, Lyon, France) at 24 ± 1°C (17). The animals had free access to drinking water and a pathogen-free rodent diet (diet A; Kliba, Basel, Switzerland). The tumors were initiated from cultured ZR-75-1 cells obtained by differential centrifugation of cell homogenates in HEPES buffer (20 mM, pH 7.4) containing 250 mM sucrose, 0.1 mM EDTA, 1 mM benzamidine, 50 μg/ml soybean trypsin inhibitor, 1 μM leupeptin, 0.1 μM aprotinin, and 1 μM pepstatin A. The membranes were incubated with 125I-labeled recombinant human IGF-I (50,000 cpm; Amersham) for 1 h at 37°C in 30 mM HEPES containing 1 mg/ml BSA, 1 mM benzamidine, and 1 μg/ml leupeptin. Nonspecific binding of 125I-IGF-I was determined in the presence of 100 nM unlabeled IGF-I. Incubations were terminated by the addition of 4 ml ice cold tris-buffered saline (10 mM, pH 7.4) and rapid filtration using Millipore HVLP 02500 filter discs.

Growth of ZR-75-1 Cells Exposed to Octreotide in Vitro. Under serum-free conditions, ZR-75-1 cells were exposed for 48 h to insulin alone or insulin in combination with either of the two somatostatin analogues, octreotide and RC-160 (Fig. 3). Compared to control cells, insulin alone (5 μg/ml) increased the cell count by 19%. When ZR-75-1 cells were treated with combinations of insulin (5 μg/ml) and the respective somatostatin

RESULTS

Effect of Continuous Octreotide Infusion on the Growth of Solid ZR-75-1 Tumors in Nude Mice. Following the finding by Nelson et al. (5) that octreotide inhibited the growth of ZR-75-1 human breast tumor cells in vitro, we established this tumor line as a transplantable solid tumor model in nude mice, in order to characterize the potential antitumor effect of octreotide in vivo. As shown in Fig. 1, continuous infusion of octreotide (10 μg/kg/h) induced a marked inhibition of solid tumor growth. During a 4-week treatment period, the inhibition of tumor growth was highly significant as compared to vehicle-treated controls (P < 0.01 for the weekly tumor size measurements). After 4 weeks of treatment, mean tumor load in octreotide-treated animals was 36.7% of that of vehicle-treated control mice.

The body weight of the animals was similar in both groups during treatment; mean control weight when treatment was terminated (4 weeks) was 21.3 ± 2 (SD) g (n = 12) as compared to 20.2 ± 2.7 g (n = 14) in the octreotide group. A plasma level of octreotide of 5.73 ± 1.6 (SE) ng/ml (n = 5) was determined on day 7 of the treatment period in continuously infused (10 μg/kg/h octreotide) ZR-75-1 tumor-bearing nude mice as determined by radioimmunoassay in a separate experiment.

Distribution of Somatostatin Receptors in ZR-75-1 Tumors. Cryosections of ZR-75-1 tumors were exposed to 125I-Tyr3-octreotide in order to visualize, by means of autoradiography, the distribution of somatostatin receptors in ZR-75-1 tumors (Fig. 2). Eight of the 12 tumors studied were shown to express specific high-affinity somatostatin receptors. The radioiodinated 125I-Tyr3-octreotide was quantitatively displaced from its binding sites on ZR-75-1 tumor tissue by coincubation with an excess of unlabeled octreotide (Fig. 2, bottom).

Fig. 1. Antiproliferative action of continuously infused octreotide in nude mice bearing solid ZR-75-1 tumors. Octreotide was administered at a rate of 10 μg/kg/h using Alzet minipumps. ◦, control; ○, octreotide treatment. Points, means (bars, ±SE) of two independent experiments; n = 12 in the control and n = 14 in the octreotide group. *P < 0.01, **P < 0.001.
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analogue, a dose-dependent decrease in cell number was observed for both analogues (Fig. 3). As illustrated in Fig. 4, a growth inhibitory action of octreotide was also demonstrated for ZR-75-1 cells exposed to IGF-I (10 nM) or EGF (1 nM) instead of insulin as the growth stimulus. The inhibition achieved by treatment with 10 pM octreotide in the presence of IGF-I and EGF was 63.9 and 60.8%, respectively of the cell count determined with the growth factor alone (Fig. 4). The corresponding effects obtained with 10 pM RC-160 in the presence of IGF-I and EGF were 65.5 and 67.7%, respectively.

Further studies on the antiproliferative effect of somatostatin analogues were performed in ZR-75-1 cells cultured in serum-containing media. Exposure of ZR-75-1 cells to octreotide for 48 h in medium containing 5% FCS as the mitogenic stimulus led to significant growth inhibition (Fig. 5). Changing the octreotide-containing medium after 24 h or simply adding stock solutions of the test compound at that time gave similar results (Fig. 5).

In contrast, the human breast cancer cell line MDA-MB-231 was unresponsive to octreotide treatment (data not shown).
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This insensitivity was correlated with the lack of specific somatostatin receptors as shown by radioligand-binding assays and receptor autoradiography of MDA-MB-231 tumors.

Receptor-binding Studies using ZR-75-1 Cells. In order to investigate whether the growth modulatory effects of various peptide factors such as EGF, IGF-I, and octreotide correlate with the presence of the respective cell surface receptors, radioligand-binding assays were performed using intact cells or membrane preparations and the radioligands [125I]-EGF, [125I]-IGF-I, and [125I]-Tyr³-octreotide.

Intact cells were used to evaluate the binding of somatostatin analogues. The concentration dependence of radioligand binding to ZR-75-1 cells is shown in Fig. 6A. The radioligand bound with high affinity to intact ZR-75-1 cells (Table 1). The binding data were best fitted by a one-site model indicating the presence of a single class of high-affinity binding sites; accordingly, Scatchard transformation of the binding data was linear.

To further characterize the structural requirements of somatostatin analogue binding to ZR-75-1 cells, competitive inhibition studies were performed using [125I]-Tyr³-octreotide as radioligand and somatostatin-14, octreotide, and [Lys-ol]₈-octreotide as competitors (Fig. 6B). The displacement of the radioligand by octreotide and somatostatin-14 was concentration dependent and maximal at 10⁻⁷ M. [Lys-ol]₈-octreotide exhibited an 50% inhibition concentration value in the binding assay that was 2 orders of magnitude smaller than that of octreotide. The 50% inhibition concentration values obtained for the three compounds were calculated to be 0.6 (octreotide), 0.8 (somatostatin-14), and 100 nM ([Lys-ol]₈-octreotide). All analogues tested inhibited the binding of [125I]-Tyr³-octreotide to the same level of nonspecific binding. Octreotide and somatostatin-14 are very potent inhibitors of growth hormone release from pituitary cells, whereas [Lys-ol]₈-octreotide is only weakly active in this functional assay (16). This indicates that the binding properties of somatostatin receptors on ZR-75-1 cells are comparable to those already characterized in pituitary cells.

In saturation experiments performed with [125I]-EGF, intact ZR-75-1 cells were incubated with increasing concentrations of the radioligand. The assay was performed at 0°C in order to prevent receptor internalization. Under these conditions, specific saturable binding sites for EGF could be detected (Table 1). To characterize the binding sites for IGF-I, membrane preparations of ZR-75-1 cells were incubated with [125I]-IGF-I at 37°C for 1 h. Specific high-affinity binding sites for IGF-I were determined (Table 1).

DISCUSSION

This study clearly demonstrates a growth inhibitory effect of octreotide (SMS 201–995) on xenografted ZR-75-1 human breast tumors (Fig. 1). In line with this, we showed a direct antiproliferative action of octreotide in ZR-75-1 cell cultures (Figs. 3–5).
The direct inhibitory effect of octreotide on the growth of ZR-75-1 cells is apparently mediated by somatostatin receptors, since receptor-binding studies and receptor autoradiography revealed that ZR-75-1 cells in vitro and ZR-75-1 tumors in vivo are somatostatin receptor positive (Figs. 2 and 6). In contrast, we failed to show an inhibition of cell growth in somatostatin receptor-negative MDA-MB-231 human breast tumor cells, which further supports the correlation between antiproliferative action of octreotide and somatostatin receptor expression. Moreover, Setyono-Han et al. (6) demonstrated that MCF-7 cells, which are estrogen-dependent human breast tumor cells like the ZR-75-1 cells, exhibit high-affinity binding sites for somatostatin in vitro and are potently inhibited by nanomolar concentrations of octreotide.

The growth inhibitory effect of somatostatin-14 on cultured tumor cells was first described in 1982 by Mascalodo and Sherline (4) who found significant inhibition of the growth of gerbil fibroma cells and human HeLa cervix tumor cells by picomolar concentrations of somatostatin. Subsequently, the antiproliferative effect of somatostatin-14 was confirmed in various other tumor systems (5, 6). Compared to somatostatin-14, its derivative octreotide has the advantage of being less susceptible to proteolytic degradation. This leads to a >30-fold increase in the duration of action in vivo, implying an apparent superiority of octreotide over somatostatin-14 for the potential treatment of cancer. Using ZR-75-1 and MDA-MB-436 human breast cancer cells, Nelson et al. (5) found that octreotide inhibited cell growth in vitro. Our data obtained with cultured ZR-75-1 cells extend these results in that we characterized various conditions that allow for octreotide-mediated inhibition of cell growth (Figs. 3–5). The antiproliferative action of octreotide was observed not only in the presence of EGF but also when IGF-I, insulin, or fetal calf serum were used to stimulate cell growth. In the parallel receptor-binding studies (Fig. 6, Table 1), specific high-affinity binding sites for somatostatin, EGF, and IGF-I could be detected, suggesting that the growth modulatory effects observed were mediated via these receptors. Moreover, the in vitro data suggest that octreotide interferes with a common pathway of the mitogenic signaling of the growth factors tested.

Regarding the mechanism underlying the receptor-mediated antiproliferative action of octreotide, it has been proposed that somatostatin acts by activating a phosphotyrosine phosphatase (18, 19). This enzyme catalyzes the dephosphorylation of phosphotyrosine residues in proteins of the signal transduction cascade, e.g., the EGF receptor, thereby abrogating the action of tyrosine kinase-dependent events. Clearly, more studies are required to further explore the significance of this mechanism for octreotide-induced tumor growth inhibition.

However, the inhibition of ZR-75-1 tumor growth in nude mice by octreotide may additionally be due to indirect effects. It has been shown previously that treatment of animals with somatostatin or its analogues induces decreased plasma concentrations of growth factors such as IGF-I (20). Moreover, the indirect inhibitory action of octreotide may involve a reduction in the levels of pituitary hormones, including growth hormone and prolactin. The decreased availability of paracrine and endocrine growth stimuli may contribute to the inhibition of tumor growth. In addition, the anticancer action of octreotide may be related to its angiostatic properties which interfere with tumor-induced blood vessel formation (12).

Using continuous infusion of octreotide (10 μg/kg/h), we could induce highly significant inhibition of the growth of ZR-75-1 breast tumors. This infusion rate was selected following the demonstration in pilot experiments that a lower infusion rate (1 μg/kg/h) led to only weak inhibitory effects (data not shown). The plasma level (5.7 ng/ml) produced by infusion of 10 μg/kg/h is well above the level (approximately 0.2 ng/ml) required for half-maximal inhibition of growth hormone secretion in various species (21).

In line with our in vivo data, Weber et al. (11) showed that octreotide, when administered two times a day, significantly inhibited the growth of MCF-7 tumors in nude mice. The effects described by these authors were rather moderate, which may be explained by the fact that they started treatment when the tumors were already far advanced (100 mm³). Using the ZR-75-1 model, we recently showed that discontinuous administration of octreotide two times a day is also efficacious in inhibiting tumor growth.

The utility of octreotide as an antitumor agent has also been confirmed by studies of different types of experimental cancer (7, 22, 23). Octreotide treatment over several months was shown to inhibit the growth of transplanted Dunning R3327-H prostate tumors in the rat (7). Another study using a rat pancreatic tumor model clearly demonstrated that octreotide inhibits the growth of an acinar cell carcinoma of the pancreas (23). After 14 days of treatment with octreotide (40 μg/kg/day), tumor weight and protein, RNA, and DNA content were decreased by 50–60%. In addition, octreotide induced a marked reduction in tumor amylase and chymotrypsin content. A high density of receptors was detected by autoradiography in the neoplastic cell area but not in the tumor stroma. The distribution of receptors was quite similar in octreotide-treated and untreated tumors, indicating that no desensitization or internalization of somatostatin receptors occurred under octreotide treatment (23).

In summary, all data presented here are consistent with the notion that the somatostatin analogue octreotide acts as an antitumor agent. This conclusion is supported by studies in tumor-bearing nude mice and in ZR-75-1 cell cultures. The effect of octreotide on tumor cell growth apparently involves direct antiproliferative effects mediated by specific somatostatin receptors, possibly via activation of phosphotyrosine phosphatases and modulation of second-messenger systems. In addition, although not addressed in this study, indirect effects involving modulation of growth factor levels, suppression of the secretion of hormones (growth hormone, prolactin, insulin, etc.), and inhibition of angiogenesis (12) may contribute to the overall inhibition profile of octreotide.

REFERENCES


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