Direct Inhibitory Effects of Somatostatin (Analogues) on the Growth of Human Breast Cancer Cells

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ABSTRACT

Various hormones and growth factors are involved in the growth regulation of breast (tumor) cells. In this report we show for the first time that an analogue of the neuropeptide somatostatin (Sandostatin) can also influence the proliferation of human breast cancer cells (MCF-7), namely, in an inhibitory fashion. With respect to dose-response relationship a bell-shaped curve was observed with the maximal inhibition of tumor cell growth at a sharply defined amount of Sandostatin (10 nM). The same effects were found with the natural hormone somatostatin-14 and another analogue (CGP 15-425). These results, together with the observation that high affinity binding sites for an iodinated derivative of Sandostatin are present in MCF-7 cells, support the conclusion that somatostatin and analogues act directly on breast cancer cells.

INTRODUCTION

Different steroid hormones (estrogens, progestins, androgens, glucocorticoids), peptide hormones (prolactin, growth hormone, insulin, calcitonin), growth factors (epidermal, transforming, and insulin-like growth factors), and other trophic substances (iodothyronines, vitamin D, retinoids) are involved in the growth regulation of breast cancer cells. Most of these factors are derived from endocrine glands such as the pituitary, gonads, and adrenals. Treatment of metastatic breast cancer is designed to decrease plasma concentrations of these hormones and factors or to antagonize the biological effects of these trophic substances directly at the level of the tumor cells.

Endocrine therapy of breast cancer consists of a variety of both medical and surgical treatment modalities including oophorectomy and hypophysectomy. Chronic treatment with analogues of LHRH, a hypothalamic hormone, causes a "partial hypophysectomy" and medical castration resulting in breast tumor regression in about 40% of premenopausal patients. Recently, neuropeptides of another class, analogues of somatostatin, have become available. Somatostatin or its analogues appear to cause suppression of the secretion of a number of pituitary and gastrointestinal hormones (18-27) such as GH and insulin. In addition, it has been reported that treatment with somatostatin analogues can decrease plasma concentrations of some growth factors such as somatomedin C (i.e., IGF-1) (25) and EGF (28). IGF-1 and EGF may also function as autocrine or paracrine growth factors for MCF-7 human breast cancer cells (8-10). The possibility of a 'complete medical hypophysectomy' by means of chronic treatment with potent long-acting somatostatin analogues in combination with other drugs [LHRH analogues, (anti)steroids, prolactin inhibitors] makes this class of peptides of potential value in the treatment of breast cancer. Furthermore somatostatin or somatostatin-like material (20-22, 29) and/or somatostatin receptors (30-32) have been found in tumors from several origins suggesting the possibility of direct effects of somatostatin on tumor cells. It has been shown that analogues of somatostatin are able to inhibit the growth of a number of classical endocrine (25-27, 33-37) and nonendocrine tumors (37-39). Somatostatin has endocrine effects, but it is believed that somatostatin can act locally as a paracrine or autocrine regulator of cell proliferation. Until now somatostatin has not been recognized as a hormone involved in the regulation of breast (tumor) cell growth. We have therefore studied whether the growth of human breast tumor cells can be influenced directly by an analogue of somatostatin. This is the first report showing that a somatostatin analogue inhibits the growth of human breast cancer cells.

MATERIALS AND METHODS

Chemicals and Materials. Estradiol was obtained from Merck, Darmstadt, West Germany. Nonradioactive Sandostatin (SMS 201-995; D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol) and 125I-labeled Tyro3, Sandostatin (1770 Ci/mmol) were kindly provided by Sandox, Basel, as was CGP 15-425 [cyclo-(Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Gaba)] by Ciba-Geigy AG, Basel, Switzerland. Somatostatin-14 (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Cys) was purchased from Sigma, St. Louis, MO. All media, sera, and antibiotics used were purchased from Grand Island Biological Co. (Breda, The Netherlands). Insulin was obtained from Organon, Oss, The Netherlands.

Cell Culture. MCF-7 human breast cancer cells in their 219th passage were originally obtained from E.G. and M. Mason Research Institute, Worcester, MA. Cells were passaged weekly and were grown in plastic T-75 flasks in RPMI 1640 medium containing 5 µg/ml phenol red and supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), insulin (10 µg/ml), and 10% fetal calf serum that had been inactivated for 30 min at 56°C (complete growth medium). For experiments, cells growing exponentially were removed by treatment with 0.1% trypsin and 3 mM EDTA in 1 ml Dulbecco's phosphate-buffered saline free of Ca2+ and Mg2+ for 10 min at 37°C and were plated in T-25 flasks in 5 ml complete growth medium at a density as indicated in the legends to the figures. The following day the monolayers were washed twice with 5 ml 0.9% NaCl and 5 ml experimental medium were added. Experimental medium consisted of RPMI 1640 medium containing phenol red with or without somatostatin (analogues), estradiol, and/or insulin, and was supplemented with 5% fetal calf serum that had been inactivated and depleted of steroids by two 45-min incubations at 50°C with dextran-coated charcoal (1% charcoal-0.1% dextran). Estradiol was added from a concentrated stock solution in absolute ethanol. In medium without estradiol the same amount of ethanol was added (<0.01%). Other vehicle controls consisted of 0.9% NaCl solution. Medium containing the respective supplements, including somatostatin (analogues), was refreshed as indicated in the legends to the figures. When indicated in the text RPMI 1640 medium without phenol red was used in the experimental medium.

Harvesting of Cells. Monolayer cells were harvested following two washes with 0.9% NaCl either by a 30 min incubation at 60°C in 1 ml NaOH or by incubation with 0.1% trypsin and 3 mM EDTA in Dulbecco's phosphate-buffered saline free of Ca2+ and Mg2+ for 10 min at ambient temperature. DNA and protein contents of the cell lysates...
were estimated by a fluorimetric method as described before (40) and according to Bradford (41), respectively. Cells harvested by trypsinization were counted in a hemocytometer after preparing a single cell suspension by repeatedly forcing the cells through a 0.6-mm needle.

Determination of Sandostatin Binding Sites by Scatchard Analysis. MCF-7 cells were cultured in experimental medium in the absence of estradiol and in the presence of insulin. On day six, after medium refreshment every second day, monolayer cells were washed twice with RPMI 1640 medium containing 0.5% bovine serum albumin. After two subsequent incubations for 1 h at 37°C in medium with the same composition the cells were harvested with a rubber policeman and resuspended in ice-cold medium. Following centrifugation for 5 min at 100 x g, the cells were resuspended in a receptor consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) with 5 mM MgCl₂, 10 mg/ml bovine serum albumin, 0.02 µg/ml phenylmethylsulfonyl fluoride, 0.02 µg/ml Bacitracin, and 200 Kallikrein inhibiting units/ml Trasylol. Cells (3 x 10⁵) were aliquoted in 1.5 ml conical polypropylene tubes and incubated with [³¹P]-labeled Tyr³-Sandostatin (specific activity, 1770 Ci/mmol) ranging from 25 to 500 pm, in the absence and presence of a 200-fold excess nonlabeled Sandostatin to correct for nonspecific binding. After incubation for 2 h at 22°C, which appeared optimal in initial experiments with cells in suspension, the cells were pelleted by centrifugation for 5 min at 13,000 x g. Following one wash with 1 ml receptor buffer, radioactivity in the pellet fractions was estimated by gamma counting (efficiency, 88%).

Statistical Analysis. Statistical analysis was performed by the non-parametric method of Wilcoxon.

RESULTS

In initial experiments MCF-7 mammary tumor cells were cultured in medium supplemented with 5% steroid-depleted fetal calf serum and with increasing concentrations of the somatostatin analogue Sandostatin both in the absence and the presence of estradiol and/or insulin. Tested under these four conditions, Sandostatin at a concentration of 10 nM resulted in a significant inhibition (P < 0.05) of the growth of the cell cultures (Fig. 1). This inhibition was most profound when the cultures were grown in medium with insulin and without estradiol. Under this condition there was an 88% inhibition of cell growth based on DNA content of the cultures (Fig. 1C). Interestingly, maximal suppression of the growth of the cultures was obtained at a sharply defined amount of Sandostatin (10 nM); at lower and higher dosages the inhibition of cell growth was less striking. The known stimulating effects of estradiol and insulin on MCF-7 cell growth were confirmed from the differences in the amounts of DNA in the cultures grown in the absence of Sandostatin (Fig. 1, left columns). To evaluate whether the growth-inhibiting effect of Sandostatin also occurs under "complete estrogen-deprived" conditions, experiments were performed in medium lacking phenol red which was shown to have weak estrogenic effects (42). In such medium without phenol red and estrogens, 10 nM Sandostatin resulted in 25 ± 3% (SE; n = 6) inhibition of MCF-7 cell growth in the presence of insulin (P < 0.02).

In subsequent experiments with medium containing phenol red without estradiol and with insulin, the inhibiting effect of Sandostatin on MCF-7 cell proliferation appeared reproducible. It was found that in addition to its effect on the DNA content, Sandostatin at a concentration of 10 nM also caused a maximal decrease in the cell number and in the protein content of the cultures (Fig. 2). Based on DNA content of the cultures from three consecutive experiments, the mean inhibition of cell growth caused by the two most effective concentrations of Sandostatin were 44 ± 1% at 5 nM and 73 ± 18% (n = 3) at 10 nM Sandostatin.

In in vivo studies using dimethylbenzanthracene-induced mammary tumors in rats, we have also observed a bell-shaped curve of dose-response relationship, showing a critical concentration of Sandostatin which causes optimal inhibition of tumor growth. Rats were treated twice daily with five different dosages (0, 0.05, 0.2, 1, 5, and 20 µg) of Sandostatin for 3 weeks. A dose of 2 x 0.2 µg/day appeared to result in maximal inhibition (83%) of tumor growth. Dosages of Sandostatin above and
**SOMATOSTATIN AND BREAST CANCER**

Fig. 2. Effects of increasing concentrations of Sandostatin on the proliferation of MCF-7 breast cancer cells. Cells were plated at a density of $4 \times 10^4$ cells/T-25 flask and were cultured for 6 days with medium refreshment every second day in the absence of estradiol and in the presence of added insulin (10 \( \mu \)g/ml). Values are means ± SD (bars) of 6-fold incubations. All doses of Sandostatin used caused significant (P < 0.05) inhibition of growth of cell cultures when expressed per cell number (A), DNA content (B), and protein content (C). *, statistically significant difference (P < 0.05) from its left neighbor.

Fig. 3. Kinetics and dose-response relationships of Sandostatin on the proliferation of MCF-7 breast cancer cells. Cells were plated at a density of $2 \times 10^4$ cells/T-25 flask and were cultured with medium refreshment daily. Experimental medium consisted of RPMI 1640 supplemented with 5% steroid-depleted fetal calf serum and 10 \( \mu \)g/ml insulin. Cultures were grown in the absence (a) or presence of 5 \( \times \) 10\(^{-9}\) (b), 1 \( \times \) 10\(^{-8}\) (c), and 5 \( \times \) 10\(^{-8}\) M (d) Sandostatin. For 6 consecutive days the cells were harvested and counted as described in “Materials and Methods.” Values are means ± SD (bars) of triplicate incubations.

Fig. 4. Scatchard plot of \(^{125}\)I-labeled Tyr\(^3\)-Sandostatin binding to MCF-7 breast cancer cells. Cells cultured as described in “Materials and Methods” to near confluency were incubated in suspension with increasing concentrations of \(^{125}\)I-labeled Tyr\(^3\)-Sandostatin (25–500 pM) in the absence and presence of a 200-fold excess nonlabeled Sandostatin. Specifically bound \(^{125}\)I-labeled Tyr\(^3\)-Sandostatin is plotted.

**DISCUSSION**

In this report we show for the first time that somatostatin and two of its analogues can inhibit the growth of breast cancer cells. Because of the observed specific high-affinity binding of the Sandostatin derivative and the observation that a narrow concentration range of Sandostatin exists in which maximal inhibition of cell proliferation is achieved, it is tempting to speculate that within 1 week desensitization may occur by chronic administration of Sandostatin dosages above 10 \( \mu \)g as a result of downregulation of the somatostatin receptors. Recently it has been observed that desensitization of normal GH-secreting cells to Sandostatin can occur within 6–10 days in a dose-dependent manner (27). A similar phenomenon was observed with other somatostatin analogues (33, 39) and with respect to insulin secretion (25, 45). Preliminary experiments in our laboratory show indeed disappearance of the specific binding sites for \(^{125}\)I-labeled Tyr\(^3\)-Sandostatin after culturing of MCF-7 cells for 6 days in the presence of Sandostatin (data not shown).

With regard to mechanism of action, somatostatin can act on mammary tumor cells by different ways. Somatostatin may exert its action by directly interacting with its specific receptor or by modulation of other receptors. Indirectly, it may act by decreasing the secretion of pituitary hormones and growth factors such as somatomedin C/IGF-1 and EGF. With respect to indirect actions of somatostatin via GH, interesting observations were made by Murphy et al. (46). Their results indicated that human GH may be a potent ligand for the lactogenic receptor in human breast cancer cells *in vitro*. Furthermore...
SOMATOSTATIN AND BREAST CANCER

Shiu and Iwasio (47) showed induction of specific proteins by GH and prolactin in human breast cancer cells. The observation of increased plasma GH levels in breast cancer patients (48) may be of importance. Somatostatin may also act as a paracrine or autocrine regulator of tumor cell proliferation or by influencing secretion of autocrine or paracrine growth factors. The interaction of mammogenic peptide hormones (GH, prolactin, and insulin), steroids, and EGF with modulation of their respective receptors (27, 49–54) in addition to the possible inhibiting effects of somatostatin on the secretion of EGF and somatomedin C, suggests a very complex mechanism through which somatostatin acts in vivo and in vitro. Antagonizing effects of estradiol and somatostatin (analogues) at the level of autocrine growth factor secretion, for instance, somatomedin C, might explain our observation that the growth inhibiting effect of Somastostatin is less pronounced when estradiol is added to the medium.

In conclusion, proliferation of MCF-7 human breast cancer cells in vitro is inhibited by somatostatin (analogues) through direct action on the tumor cells. It is interesting to note that analogues of LHRH (14, 55–58) and somatostatin (this report) have direct inhibitory effects on tumor cell growth antagonizing in vitro especially the biological effects of those steroid (as estradiol by LHRH analogues) and peptide hormones (GH and insulin by somatostatin analogues) in which secretion in vivo is suppressed by pharmacological doses of the same neuropeptide analogues.

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