Somatomedin-C Receptors and Growth Effects in Human Breast Cells Maintained in Long-Term Tissue Culture

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ABSTRACT

Somatomedin-C (SM-C) is a growth hormone-dependent polypeptide with potent mitogenic activity in vivo and in vitro. In the present study, we show that four human breast cell lines maintained in long-term tissue culture (MCF-7, T47-D, MDA-MB-231, and HBL-100) have type I somatomedin receptors and that SM-C stimulates DNA synthesis in these cells. The concentration of SM-C required for half-maximal stimulation of DNA synthesis varied from 0.03 nm in the MCF-7 cell line to 0.6 nm in the T47-D cells. Porcine insulin also stimulated DNA synthesis in all cell lines but, compared to SM-C, 10- to 1000-fold-higher concentrations were required.

SM-C receptors on the four breast cell lines were characterized by competitive binding and chemical cross-linking techniques. The four cell lines varied widely in their SM-C binding. In three of the cell lines (MCF-7, MDA-MB-231, and HBL-100), the SM-C receptor had a Kd for SM-C of 0.5 to 1 nm, and insulin competed for binding but with a potency 1/10 to 1/100 that of SM-C. In the T47-D cell line, the Kd for SM-C binding was 4 nm, and insulin competed poorly for binding. Chemical-cross-linking studies showed that all four cell lines have type I somatomedin receptors. Variations in the sensitivity to SM-C and insulin stimulated DNA synthesis in the MCF-7 and T47-D cell lines correlated with type I somatomedin receptor binding by these cells.

The data indicate that SM-C is mitogenic for cultured human breast cells and are consistent with the hypothesis that the mitogenic effect of insulin for these cells is mediated through the type I somatomedin receptor.

INTRODUCTION

A number of hormones have been shown to affect the growth of human mammary tissue. These include steroid hormones, such as estrogens (19) and corticosteroids (20), and protein hormones, such as insulin (24) and epidermal growth factor (25). The effects of the growth hormone-dependent serum growth factors (the SMs) on mammary cell growth have not, however, been reported. The present study was undertaken to investigate such effects.

Two SMs have been purified from human serum. These are SM-C (34) [which is identical to Insulin-like Growth Factor I (17, 28)] and IGF-II (29). These peptides show considerable sequence homology with both proinsulin and each other. SM-C is a basic peptide (pl 8.2), the serum concentration of which shows strong growth hormone dependence (10). IGF-II is a neutral peptide (pl 7.0), of which the serum level is less growth hormone dependent (36). Although both SM-C and IGF-II are mitogenic for cultured cells, SM-C appears to have more potent mitogenic effects in vivo (32).

Like other polypeptide hormones, the SMs are believed to act by first binding to specific cell membrane receptors. For the SMs, 2 physicochemically distinct receptor types have been identified. The type I SM receptor is a heterotetramer composed of 2 sets of nonidentical subunits held together by interchain disulfide bonds (15, 21, 33). This receptor is structurally similar to the insulin receptor, and the 2 receptors may be evolutionarily related (31). The intact type I receptor has molecular weight in excess of 330,000. Upon reduction, a binding subunit with a molecular weight of approximately 130,000 has been identified (15, 21, 33). The type I receptor binds SM-C somewhat better than IGF-II. Insulin, at high concentrations, also binds to this receptor type (15, 21). The type II SM receptor is a single-chain molecule with a molecular weight of 220,000 (15, 21). This receptor binds IGF-II better than IGF-I. Insulin does not bind to the type II receptor (15, 21).

The present studies were undertaken to determine if SM-C is mitogenic for cultured human breast cells and to characterize the SM receptors on these cells. An additional goal of this study was to determine if the mitogenic effect of insulin on these cells is mediated through the SM-C receptor.

MATERIALS AND METHODS

Materials. Human SM-C was purified from Cohn Fraction IV-1 as described previously (9). The material used in these studies had a specific activity of 7500 units/mg and was judged to be greater than 80% pure (9). For determination of nonspecific binding, a partially purified SM-C preparation (4% pure) was used. Porcine insulin was a gift of Dr. Mary Root (Eli Lilly Co., Indianapolis, IN). SM-C and insulin were iodinated to specific activities of 100 to 200 μCi/μg using a modified chloramine T procedure (9). The SM-C-deficient human serum used in these studies was a single pooled sample obtained from individuals with documented growth hormone deficiency, who had not received growth hormone for at least 3 months prior to serum collection. This sample contained 8 ng of immunoreactive SM-C/ml (normal range, 100 to 180 ng/ml). All serum used in these studies was dialyzed twice against 10 volumes of phosphate-buffered saline, filter sterilized, and stored at −20°.

Cell Culture. The T47-D, MDA-MB-231, and HBL-100 human breast cell lines were obtained from the EG & G Mason Research Institute through the auspices of the Breast Cancer Task Force of the National Cancer Institute. The MCF-7 cell line (passage 230) was a gift of Dr. W. McGuire, University of Texas Health Science Center, San Antonio, TX. All cell lines were grown in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium containing NaHCO3 (1.2 g/liter), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 15 mg and 5% FBS. Cells were maintained at 37° in a humidified atmosphere of 5% CO2, 95% air and were split twice weekly to maintain log phase growth. Cells were monitored for Mycoplasma contamination at monthly intervals (4).
DNA Synthesis. As a measure of DNA synthesis, [³H]thymidine incorporation into acid-insoluble material was measured. In these experiments, the cells were plated into 16-mm cluster plates (3 to 4 x 10⁴ cells/well) in medium containing 1% FBS. After 16 to 20 hr, the medium was removed and replaced with medium containing 0.2% FBS. After a period of serum restriction varying from 48 to 72 hr, this medium was removed, and 0.5 ml of medium containing 0.1% BSA, penicillin (10 units/ml), streptomycin (10 units/ml), and varying concentrations of hormones and/or serum was added. After 16 to 20 hr (30 hr for the T47-D cell line), 0.5 μCi of [³H]thymidine (5 Ci/mmol; Amersham, Arlington Heights, IL) in a volume of 25 μl was added to each well. After an additional 2-hr incubation, the wells were aspirated and washed once with 0.5 ml of phosphate-buffered saline, twice with 0.5 ml of 10% trichloroacetic acid, and once with 0.5 ml of methanol. All washes were performed at 4°C. Precipitated material was solubilized by incubating with 0.5 ml of 0.5 N NaOH for 30 min at 37°C. After neutralization, the contents of each well were mixed with 5 ml of scintillation fluid (ACS II; Amersham) and radioactivity was quantitated on a Packard Tri-Carb liquid scintillation counter with an efficiency of 60%. In all experiments, 100% stimulation was arbitrarily defined as the difference in acid-insoluble counts between cultures treated with 5% FBS and those treated with medium containing only 0.1% BSA.

Binding of [¹²⁵I]-labeled SM-C to Cultured Cells. Binding studies were performed using monolayer cultures of cells. To limit possible artifacts due to variations in cell synchrony or density-dependent variations in SM-C binding, all binding studies were performed using log phase cells at final densities of 0.6 to 1.0 x 10⁶ cells/well. The cells were plated into 35-mm cluster plates in medium containing 5% FBS. After 72 ± 4 hr, the medium was removed, and the monolayer was washed once with phosphate-buffered saline. One ml of medium containing 0.1% BSA, [¹²⁵I]-labeled SM-C (20 to 200 pm), and the unlabeled polypeptide as indicated was then added to triplicate wells. After incubation for the desired time and temperature (usually 90 min at 4°C), the medium was removed, and the monolayer was washed twice with phosphate-buffered saline at 4°C. The monolayer was solubilized with 0.5 N NaOH, and an aliquot was removed to determine bound radioactivity. Nonspecific binding was defined as the radioactivity remaining bound in the presence of excess unlabeled SM-C (100 nM). Binding experiments were repeated a minimum of 3 times with each individual cell line.

Chemical Cross-Linking of [¹²⁵I]-labeled SM-C and [¹²⁵I]-labeled Insulin to Cultured Cells. Cross-linking studies were performed using a modification of the method of Adams et al. (1). The cells were plated into 35-mm culture plates in medium containing 5% FBS and used 2 to 3 days later. The monolayer was washed once with phosphate-buffered saline, and 1.0 ml of medium containing 0.1% BSA, 3 to 10 nM iodinated hormone, and unlabeled hormones as indicated was added. Following incubation (90 min at 4°C), the monolayer was washed once with ice-cold phosphate-buffered saline, and 1.0 ml of a freshly prepared solution of 0.1 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) in buffer containing 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.12 M NaCl, 5 mM KCl, and 8 mM glucose, pH 8.0, was added. After a 15-min incubation at 22°C, this solution was aspirated, and the reaction was quenched by adding 2.0 ml of 10 mM Tris, pH 7.4, for 5 min. The monolayer was solubilized by addition of 0.5 ml of 0.3% SDS in 0.06 M Tris-HCl, pH 6.8, with or without 100 mM dithiothreitol. All samples were boiled for 3 min immediately prior to electrophoresis.

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Chart 1. DNA synthesis measured as [³H]thymidine incorporation into acid-insoluble material by cultured human breast cells. Cells were plated into 16-mm culture dishes in medium containing 1% FBS. After 16 to 20 hr, the medium was removed and replaced with medium containing 0.2% FBS. Seventy-two hr later, this medium was removed and replaced with the test medium, as indicated. After 16 to 20 hr (30 hr for the T47-D cell line), the cells were pulse labeled with 0.5 μCi of [³H]thymidine for 2 hr and the wells were then processed as described in Materials and Methods. One hundred % stimulation was defined as the difference in acid-insoluble counts observed between cultures treated with 5% FBS and those receiving serum-free medium containing 0.1% BSA. Cells were exposed to SM-C (20 ng/ml), 5% SM-C-deficient serum (HX), porcine insulin (10 μg/ml; INS), 5% SM-C-deficient serum plus SM-C (20 ng/ml) (HX + SM-C), or 5% SM-C-deficient serum plus insulin (10 nM) (HX + INS). Values are the mean of triplicate wells. S.D.s were less than 10% of the mean maximal counts incorporated. Cell counts, performed on replicate plates, showed no significant differences among any of the treatment groups.
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SDS-polyacrylamide gel electrophoresis (0.05% SDS) was performed using the discontinuous buffer system of Laemmli (18). The gels were stained with 0.25% Coomassie Blue R250 in methanol:acetic acid, destained, dried, and autoradiographed with Kodak X-O-mat film (Eastman Kodak, Rochester, NY) using a DuPont Cronex Lightning-plus enhancing screen (DuPont Instruments, Wilmington, DE) at -70°.

RESULTS

Effect of SM-C and Serum Components on [3H]Thymidine Incorporation by Cultured Human Breast Cells. To determine if SM-C is mitogenic for cultured human breast cells, its effect on DNA synthesis following a period of serum restriction was investigated. Because of previous reports indicating that, in certain cell types, SM-C stimulates DNA synthesis only in the presence of other non-growth hormone-dependent serum components (1, 27), the effect of SM-C on DNA synthesis in the presence and absence of 5% SM-C-deficient serum was evaluated. As shown in Chart 1, SM-C, at a concentration of 20 ng/ml (2.6 nm), stimulated DNA synthesis in all 4 cell lines. In all 4 cell lines, 5% SM-C-deficient serum also stimulated DNA synthesis. In 2 of the cell lines (T47D and MDA-MB-231), the effects of SM-C and SM-C-deficient serum appeared additive, whereas in the MCF-7 cell line, the combination was consistently less effective than that observed when either SM-C or SM-C-deficient serum was added alone. Chart 1 also shows the effect of porcine insulin (10 μg/ml) on [3H]thymidine incorporation by these cells. In all cell lines, the effect of insulin either alone or in combination with SM-C-deficient serum paralleled the effect observed with SM-C.

The dose-response curves for SM-C- and insulin-stimulated DNA synthesis in the MCF-7 and T47-D cell lines are shown in Chart 2. In the MCF-7 cells, ED_{50} occurred at SM-C and insulin concentrations of 0.03 and 0.3 nm, respectively. These results with insulin are in good agreement with those reported by Osborne et al. (24). In the T47D cell line, the ED_{50} for SM-C was 0.6 nm, and that for insulin was 100 nm. These differences in sensitivity of the 2 cell lines to the 2 hormones parallel differences in binding of the hormones to the SM-C receptors on these cell lines as described below.

Binding of 125I-labeled SM-C and 125I-labeled Insulin to Cultured Human Breast Cells. Binding of 125I-labeled SM-C and 125I-labeled insulin to monolayer cultures of the 4 cell types is rapid, nearing equilibrium in 30 min at 37° and 90 min at 4° (not shown). Because hormone degradation was slower at the lower temperature, subsequent experiments were performed at 4°. In Chart 3, the binding of tracer concentrations of 125I-labeled SM-C to the 4 cell lines is shown. 125I-labeled SM-C binding was consistently highest for the T47-D and MCF-7 lines and less for the MDA-MB-231 and HBL-100 lines. This wide variation in binding suggested variations in SM-C receptor type, number, or affinity among the individual cell lines. Also shown in Chart 3 is the binding of 125I-labeled insulin to these cells. In all 4 cell lines, the 125I-labeled insulin binding was much lower than the 125I-labeled SM-C binding and was too low to study in detail using the experimental protocol utilized.

To characterize the SM-C receptors on these 4 cell lines, we first investigated the inhibition of 125I-labeled SM-C binding by unlabelled SM-C and by porcine insulin (Chart 4). In the MCF-7

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Chart 1. Dose-response curves for SM-C and insulin-stimulated DNA synthesis in MCF-7 and T47-D cell lines. Experiments were performed as described in the legend to Fig. 1. Points are the means of triplicate determinations. THY, thymidine.

Chart 2. Dose-response curves for SM-C- and insulin-stimulated DNA synthesis in MCF-7 and T47-D cell lines. Experiments were performed as described in the legend to Fig. 1. Points are the means of triplicate determinations. THY, thymidine.

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Chart 3. Binding of 125I-labeled SM-C (●) and 125I-labeled insulin (□) to monolayer cultures of human breast cells. Results are expressed as cpm of radiolabeled hormone bound (BRD) specifically (SPEC) per million cells. Cells were incubated with approximately 40 pm 125I-labeled SM-C (43,200 cpm) or 125I-labeled insulin (39,200 cpm) for 90 min at 4°, then processed as described in "Materials and Methods." Nonspecific binding was determined using 100 nm unlabeled SM-C or 1.73 μm unlabeled insulin and was less than 2% of the total counts added for all cell lines. Values are the means of triplicate determinations. S.D.s were less than 10% of the mean maximal binding.
cell line, half-maximal displacement of 125I-labeled SM-C binding occurred at an unlabeled SM-C concentration of approximately 0.5 nM. Insulin competed with 125I-labeled SM-C for binding to this receptor, but approximately 100 nM insulin was required for half-maximal displacement. Qualitatively similar results were observed with the MDA-MB-231 and HBL-100 cell lines, although half-maximal displacement of 125I-labeled SM-C by both hormones consistently occurred at slightly higher concentrations than those observed with the MCF-7 cell line (Chart 4). However, in the T47-D cell line, half-maximal displacement of 125I-labeled SM-C required 8- to 10-fold-higher concentrations of unlabeled SM-C (4 nM). Insulin also competed for binding to the SM-C receptor on the T47-D cells, but μM concentrations were required for half-maximal displacement (Chart 4).

**Chemical Cross-Linking of 125I-labeled SM-C and 125I-labeled Insulin to Cultured Human Breast Cells.** To further characterize the SM-C receptors on the individual cell lines, an affinity labeling technique similar to that used to characterize SM receptors in other tissues was utilized (1, 15, 21, 33). In these experiments, 125I-labeled SM-C was covalently cross-linked to its receptor using the bifunctional cross-linking reagent disuccinimidyldiisocyanate. When analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, all 4 cell lines showed one band of specific 125I-labeled SM-C binding with a molecular weight in excess of 330,000 (not shown). No band in the M, 220,000 range, characteristic of a type II SM receptor, was observed. Under reducing conditions, all 4 cell lines showed 2 bands of specific 125I-labeled SM-C binding (Figs. 1 and 2, Lanes 1 and 4). These bands had molecular weights of 130,000 and greater than 330,000. The M, 130,000 band is believed to be the binding subunit of the type I SM receptor (15, 21, 33). The larger species

![Chart 4](image-url)
Fig. 2. Autoradiogram showing the size and specificity of the 125I-labeled SM-C receptor complexes on the HBL-100 and MDA-MB-231 cells, examined after reduction with 100 mM dithiothreitol. Cells were incubated with 125I-labeled SM-C in the absence (Lanes 1 and 4) and presence of unlabeled insulin (1.7 μM; Lanes 2 and 5) or SM-C (130 nM; Lanes 3 and 6) for 90 min at 4°C and then processed as described in the legend to Fig. 1. A 3 to 15% polyacrylamide gradient was used.

probably represents receptor aggregates which are not dissociated by the dithiothreitol treatment. In the presence of porcine insulin (10 μg/ml), binding to both bands was decreased in all cell lines (Figs. 1 and 2, Lanes 2 and 5). These findings indicate that, in all 4 cell lines, 125I-labeled SM-C is binding to a type I SM receptor.

In Fig. 3 are shown the results obtained with 125I-labeled insulin was covalently cross-linked to the different cell lines. Because of the low insulin binding, much longer exposures were necessary to visualize these bands. Under reducing conditions, 2 bands of specific insulin binding with apparent molecular weights of 140,000 and greater than 330,000 were observed (Fig. 3, Lanes 3 to 6). These bands are similar to those observed for insulin receptors on rat liver and human placental membranes (Fig. 3, Lanes 1 and 2).

DISCUSSION

This paper demonstrates that SM-C stimulates DNA synthesis in 4 human breast cell lines maintained in long-term cell culture. In all 4 cell lines, physiological concentrations of the hormone stimulate DNA synthesis, suggesting that this effect may be important in vivo. Interestingly, the individual cell lines vary widely in their sensitivity to SM-C. The MCF-7 cell line is the most sensitive, with half-maximal stimulation of DNA synthesis (ED50) occurring at approximately 0.03 nM SM-C (0.2 ng/ml). By contrast, in the T47-D cell line, the ED50 is 20-fold higher (0.6 nM) and is similar to that observed for BALB/c-3T3 fibroblasts in our laboratory (approximately 0.5 nM). Although additional studies are necessary, these observations suggest that the MCF-7 cell line has an enhanced sensitivity to SM-C. The binding studies discussed below suggest that this is the result of an increased number of type I SM receptors on these cells.

The binding and chemical cross-linking experiments reported here indicate that all 4 cell lines contain type I SM receptors. In 3 of the cell lines (MCF-7, MDA-MB-231, and HBL-100), this receptor has a Keq and binding specificity similar to that of type I receptors on other cells and tissues. However, in the T47-D cell line, the SM-C receptor has an anomalously high Keq for both SM-C and insulin. Such a difference in type I SM receptor affinity between individual cell lines has not been reported previously, and its significance is unknown. It is noteworthy, however, that this difference in affinity was not associated with any apparent alteration in the size of this receptor as determined by SDS-polyacrylamide gel electrophoresis. It should be noted that the present studies do not exclude the presence of type II SM receptors on any of these cell lines. Using 125I-labeled SM-C, we have been unable to detect the type II SM receptors on either rat liver or human placental membranes (both have high concentrations of type II receptors [15, 21]).

A comparison of the dose-response curves for SM-C-stimulated DNA synthesis (Chart 2) and 125I-labeled SM-C displacement (Chart 4), although obtained under different experimental conditions, is informative. In the MCF-7 cell line, the ED50 for SM-C-stimulated DNA synthesis was 15-fold lower than the apparent Keq for type I receptor binding, and the maximum biological effect (as measured by [3H]thymidine incorporation) occurred at a SM-C concentration at which there was less than 20% type I receptor occupancy. In the T47-D cell line, these differences were less pronounced, but maximum biological effect occurred at less than 50% receptor occupancy. Such a relationship between receptor occupancy and biological effect has been observed in other hormone-receptor systems (12, 23) but has not been reported for SM-C. It has been termed the reserve

the SM-C-deficient serum may be sufficient to stimulate DNA synthesis in the MCF-7 cell line. The stimulatory effect of the SM-C-deficient serum could also be due to the IGF-II present in the sample. Although the IGF-II content of this sample was not determined, 5% SM-C-deficient serum would be expected to contain 10 to 15 ng of IGF-II/ml (1 to 2 nM) (14), and this may be sufficient to stimulate DNA synthesis in these cells. Recently, it has been postulated that de novo SM synthesis by cultured cells is important for their growth in SM-deficient serum (1). A number of studies (1–3, 6) have documented SM production by cultured human cells, including breast cells (3), and we have found that all 4 cell lines examined in this study produce immunoreactive SM-C. We have not determined, however, if this material is biologically active, or if it is produced in sufficient quantities to stimulate DNA synthesis in these cells. Finally, the stimulatory effects of the SM-C-deficient serum may be due to the presence of other non-growth hormone-dependent serum components which are mitogenic for these cells. The finding that SM-C alone does not produce optimal DNA synthesis in 3 of the 4 cell lines confirms the importance of other serum components in the growth of these cells.

In the present study, we have shown that SM-C stimulates DNA synthesis in human breast cells in vitro. Hypophysectomy is generally believed to inhibit breast tumor growth because of its effects on serum estrogen levels, but 10% of patients with estrogen receptor-negative breast tumors nevertheless respond to this form of therapy (26). An in vivo SM-C dependency could explain this observation, because hypophysectomy dramatically lowers serum SM-C levels (10). Similarly, high-dose estrogen paradoxically inhibits breast tumor growth in certain patients. High concentrations of estrogen also lower serum SM-C concentrations, apparently by antagonizing the stimulatory effect of growth hormone on SM-C generation (5). Finally, maternal SM-C levels increase during the last half of gestation (11). Such an increase could contribute to the rapid growth which occurs in some tumors during pregnancy.

In summary, we have demonstrated that 4 cultured human breast cell lines have type I SM receptors and that physiological concentrations of SM-C stimulate DNA synthesis in these cells. The mitogenic effect of insulin on these cells appears to be mediated through the SM-C receptor. These results suggest that clinical therapy designed to lower the serum SM-C concentration may be beneficial in inhibiting breast tumor growth in vivo. Although no agents which reliably block growth hormone release in patients with normal pituitary function have been found, the recent discovery of a growth hormone-releasing factor (13, 30) suggest that analogues with antagonistic activity may be developed. Such agents could prove useful as adjuncts in the treatment of patients with advanced breast cancer.

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