Receptors for D-Trp^6-Luteinizing Hormone-Releasing Hormone, Somatostatin, and Insulin-Like Growth Factor I in MXT Mouse Mammary Carcinoma (42987)

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Abstract. **Membrane receptors for D-Trp^6-luteinizing hormone-releasing hormone (D-Trp^6-LH-RH), somatostatin-14 (SS-14), and insulin-like growth factor I (IGF-I) were estimated in MXT mammary cancers of mice using sensitive multipoint micromethods. The receptors were characterized in untreated animals and following *in vivo* treatment with microcapsules of the agonist D-Trp^6-LH-RH and the somatostatin analog RC-160, which strongly inhibited tumor growth. In the control group, D-Trp^6-LH-RH was bound to the single class of saturable, specific, noncooperative receptor sites (K_d, = 29.3 ± 8.48 × 10^{-9} *M*; B_{max} = 4.55 ± 0.31 pmol/mg membrane protein). Treatment with D-Trp^6-LH-RH alone or in combination with RC-160 produced down-regulation of membrane receptors for D-Trp^6-LH-RH on MXT mammary tumor cells. RC-160 alone and ovariectomy were without effect on D-Trp^6-LH-RH receptors. On the membrane surface of MXT mammary cells, we found one class of high affinity, specific, saturable binding sites for SS-14 (K_d = 4.4 ± 1.9 × 10^{-9} *M*; B_{max} = 0.58 ± 0.21 pmol/mg membrane protein). Treatment with RC-160 alone or combined with D-Trp^6-LH-RH significantly increased both the dissociation binding constant (K_d = 18.6 ± 3.5 × 10^{-9} and 10.1 ± 0.7 × 10^{-9} *M*, respectively) and the binding capacity (B_{max} = 13.98 ± 1.7 and 21.00 ± 4.0 pmol/mg membrane protein, respectively). We also found specific binding sites (K_d = 3.01 ± 0.15 × 10^{-9} *M*; B_{max} = 2.24 ± 0.96 pmol/mg membrane protein) for IGF-I in the membrane fractions of MXT mammary cancers. Chronic treatment with D-Trp^6-LH-RH and RC-160 alone or in combination, as well as ovariectomy, significantly decreased the dissociation binding constant of IGF-I membrane receptors on MXT mammary cells. Our results strongly suggest an important role of LH-RH, SS-14, and IGF-I in the growth of MXT mammary carcinoma. Changes in characteristics of receptors after treatment with analogs of LH-RH and SS-14 along with tumor growth inhibition provide additional support for the direct effect of these peptides on tumor cells. A possible significance of these findings as applied to a clinical environment is discussed.** [P.S.E.B.M. 1989, Vol 192]

Various experimental studies suggest that analogs of luteinizing hormone-releasing hormone (LH-RH) can be used for the treatment of estrogen-dependent breast cancer (1, 2). Regression of tumor mass and disappearance of metastases in premenopausal and postmenopausal women with breast cancer treated with D-Trp^6-LH-RH, Buserelin, or Leuprolide have been previously reported (2). The inhibitory action of LH-RH analogs on tumor growth was thought to be mediated mainly by the suppression of the pituitary-gonadal axis with a resulting decrease in the circulating LH, follicle-stimulating hormone, estrogen and prolactin levels (1). However, recent receptor-binding studies revealed that LH-RH and some of its analogs can also exert a direct effect on rat and human breast cancers and breast cancer cell lines (3, 4). Somatostatin analogs such as RC-160 and SMS 201-995 reduce growth hormone (GH) and prolactin levels and may also antagonize the effect of various growth factors (5). In addition, somatostatin receptors were found in 35.6% of 500 human breast cancer samples examined (3), suggesting a direct effect of somatostatin-14 (SS-14) on human breast cancer cells. A significant synergism between the LH-RH agonists and SS-14 analogs in the inhibition of

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tumor growth was demonstrated when both peptides were given together (6). SS-14 analog RC-160 is being tried as an adjunct in combination with agonists of LH-RH (7) in the treatment of breast cancer in women.

The exact mechanism of action of LH-RH and SS-14 analogs is not yet completely understood, and it can include, in the case of SS-14 analogs, an effect on some growth factors and their receptors (5). Insulin-like growth factor I (IGF-I; somatomedin C) is the best known member of a family of insulin-like growth factors (8). Receptor binding of IGF-I was previously demonstrated in various human breast cancer lines (9) as well as in primary breast cancer cells (10).

The effects of the treatment with LH-RH agonists on androgen receptor content in human prostate cancers were previously described (11), but to our knowledge there are no studies about D-Trp^6-LH-RH receptors in human breast or prostate cancers after this treatment. The aim of this study was to define binding characteristics of D-Trp^6-LH-RH, SS-14, and IGF-I in the membranes of MXT mammary cancer cells and to investigate the changes in number and affinity of membrane receptors for these peptides after prolonged treatment with the analogs.

Materials and Methods

Peptides. D-Trp^6-LH-RH, somatostatin-14, and [Tyr^{11}]somatostatin-14 were provided by Debiopharm S. A. (Lausanne, Switzerland). Somatostatin analog RC-160, (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2), originally synthesized by solid-phase methods and evaluated in our laboratory, was made by classical synthesis by Novabiochem (Laufelfingen, Switzerland). Microcapsules of D-Trp^6-LH-RH and RC-160 were prepared by Dr. P. Orsolini at Cytotech (Martigny, Switzerland) and supplied by Debiopharm. Delayed release formulation of D-Trp^6-LH-RH in an aliquot of 36 mg maintained a continuous liberation of about 25 μg/day of the analog for 30 days. Microcapsules of RC-160 were designed to release about 25 μg/day of RC-160 for 30 days from an aliquot of 30 mg. Recombinant human IGF-I was kindly provided by Genentech, Inc. (San Francisco, CA). Reagents for radioimmunoassay of LH were provided by the National Hormone and Pituitary Program of NIDDH.

Treatment of Experimental Animals. Thirty-seven female BDF mice weighing 20 ± 1 g were housed four to five per cage at the Animal Research Facility of our institute at 72 ± 2°F and 55 ± 5% humidity. The mice were inoculated subcutaneously with 1 mm^3 of florid MXT (steroid receptor-positive) mouse mammary cancer tissue. One month after the inoculation, the tumor volumes were measured and animals were divided into groups with approximately equal tumors. The treatment was started 1 day after the first measurement of the tumor volumes. The animals were divided into the following groups: 1, controls (vehicle treated) (eight mice); 2, D-Trp^6-LH-RH treated (eight mice); 3, RC-160 treated (eight mice); 4, combination treated with both D-Trp^6-LH-RH and RC-160 microcapsules (eight mice); and 5, surgically ovariectomized animals (five mice). The tumor volumes were measured weekly until the end of the fourth week after the beginning of the treatment. The percent change in tumor volume was calculated on the basis of individual responses. All data are expressed as mean ± SE. Statistical analyses of the data were performed using Duncan's new multiple range test or Student's *t* test. At this time animals were decapitated, and the tumors were removed and weighed. About one-half of each tumor was used for receptor-binding assay.

Buffers. The homogenization buffer for all membrane receptor assays consisted of 0.3 *M* sucrose, 25 m*M* Tris base, 0.25 m*M* phenylmethylsulfonyl fluoride, 1 m*M* EGTA, 10 m*M* monothioglycerol, and Trasylol (aprotinin) at 10,000 kallikrein inactivator units/liter (pH = 7.5). The incubation buffer for the binding of IGF-I was composed of 25 m*M* Tris base, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 10 m*M* monothioglycerol, 0.25 m*M* phenylmethylsulfonyl fluoride, 100,000 units/liter of Trasylol, and 1% bovine serum albumin (BSA) (pH 7.5). For D-Trp^6-LH-RH binding experiments, the assay buffer was 25 m*M* Tris base, 1 m*M* dithiothreitol, 1 m*M* EDTA, 0.25 m*M* phenylmethylsufonyl fluoride, and 0.1% BSA (pH = 7.5). Receptor binding of SS-14 was carried out in 50 m*M* Tris base, 5 m*M* $MgCl_2$, 0.2% BSA, 100,000 units/liter of Trasylol, bacitracin (20 mg/liter), and 0.25 m*M* phenylmethylsufonyl fluoride (pH = 7.5). The wash buffer was the same as the assay buffers, but did not contain BSA. The chemicals used for buffers were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Membranes. Samples obtained from MXT mammary carcinoma specimens were weighed and divided into two portions. One portion was used for histologic examination and the other part was frozen on dry ice for receptor measurements. Crude liver membranes prepared from adult female and male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) served as negative controls for IGF-I binding. Pituitary and cerebral cortex tissue of male Sprague-Dawley rats were used as positive controls for D-Trp^6-LH-RH and somatostatin-14 binding.

Samples were cleaned of fat and connective tissue, cut into small slices, and homogenized in 5 volumes of sucrose buffer using an Ultra-Turrax homogenizer (Tissumizer, Tekmar, Cincinnati, OH) at maximal speed 5- × 5-sec strokes at 0°C. The homogenate was centrifuged at 500*g* for 10 min at 4°C. The supernatant containing the crude membrane fractions was then ultracentrifuged at 70,000*g* for 45 min at 4°C (Beckman Preparative Ultracentrifuge, Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was resuspended in wash buffer and used for the receptor-binding

studies. Protein concentration was determined by the Bio-Rad protein assay kit according to Bradford (12).

Radioiodination of Peptides. For the radioiodination of IGF-I, the chloramine-T method of Greenwood et al. (13) was modified to a mild stoichiometric procedure. An aliquot of the peptide (2 μg in 10 μl of 0.01 *N* acetic acid) was placed in a 1.5-ml polypropylene conical vial. Forty microliters of 0.5 *M* phosphate buffer (pH = 7.5) and 2 μl of $Na^{125}I$ (Amersham, Arlington Heights, IL) in a volume of 20 μl were added. The iodination was started by adding 5 μg of chloramine-T (0.5 mg/ml in 0.05 *M* phosphate buffer). The reaction was performed at 4°C for 10 min. The labeled peptide was purified by high-performance liquid chromatography using an Automated Gradient Controller, a pair of M-6000-A pumps, and a U6K injector (Waters, Milford, MA). The specific activity of the labeled compound was 105.5 μCi/μg as calculated by the isotope recovery.

For the radioiodination of D-Trp^6-LH-RH and [Tyr^{11}]somatostatin, a modification of the chloramine-T method was used. An aliquot of the peptide (5 μg in 5 μl of 0.01 *N* acetic acid) was mixed with 40 μl of 0.5 *M* phosphate buffer and 1 mCi $Na^{125}I$ in a volume of 2 μl. Ten microliters of chloramine-T were added. The procedure was performed at room temperature. The purification of the labeled peptide was carried out using the same high-performance liquid chromatography and procedure as described above, except that a C-18 column (W-Porex 5C18, 250 × 4.6 mm i.d.; Rancho Palos Verdes, CA) was employed. The specific activity was found to be 78 μCi/μg for [Tyr^{11}]SS-14 and 106.6 μCi/μg for D-Trp^6-LH-RH as calculated by the isotope recovery.

Receptor Binding of Peptides. The binding assay of D-Trp^6-LH-RH was conducted as described previously (3). Binding reactions were performed in 12- × 75-mm polypropylene conical or round bottom culture tubes at 4°C, using a competitive inhibition method. Displacement curves were obtained with 100–140 pmol of iodinated ^{125}I,D-Trp^6-LH-RH in the presence of increasing amounts of unlabeled D-Trp^6-LH-RH (160 pmol–1.6 μM). The procedure was as follows: 50 μl of unlabeled D-Trp^6-LH-RH in incubation buffer and 50 μl of labeled ligand were placed into the tube, vortexed, and 50 μl of the suspension containing the membrane receptor fraction were added. In each binding experiment, one set of tubes was used with 50 μl of wash buffer instead of membrane to determine the radioactivity bound to nonmembrane particles. Each assay point was performed in triplicate. The tubes were incubated for 60 min at 4°C. The reaction was terminated by rapid filtration through glass fiber filters (Filtermats-Receptor Binding, Skatron, As., Lier, Norway), prewetted in assay buffer, and presoaked for at least 3 hr in 0.5% polyethylenimine solution to minimize filter adsorption. A semiautomatic cell harvesting system (Skatron, As.) with 10-sec washing time was used. The radioactivity of filters was counted in an automatic gamma counter (Micromedic System, Inc., Huntsville, AL). The specificity of D-Trp^6-LH-RH binding to its receptors was investigated using competitive inhibition studies with labeled D-Trp^6-LH-RH and unlabeled SS-14 (150 pmol–1.5 μM) and IGF-I (0.1–100 ng).

The receptor binding of SS-14 was performed as described for D-Trp^6-LH-RH with some modifications. Binding reaction was performed at room temperature (21°C), using a competitive inhibition method. Displacement curves were obtained with 90–120 pmol of iodinated [^{125}I,Tyr^{11}]somatostatin-14 in the presence of increasing amounts of unlabeled SS-14 (150 pmol–1.5 μM). Incubation was performed for 120 min at room temperature (21°C). The specificity of SS-14 binding to its receptors was investigated using competitive inhibition studies with labeled SS-14 and unlabeled D-Trp^6-LH-RH (160 pmol–1.6 μM) and IGF-I (1–100 ng).

The binding assays of IGF-I were performed in a total volume at 150 μl. A competitive inhibition method was used and displacement curves were obtained with 0.4–0.5 ng of ^{125}I-IGF-I in the presence of increasing amounts of unlabeled IGF-I (0.1–100 ng). A procedure similar to that described above was used, except that the tubes were incubated for 2 hr at 21°C. The reaction was stopped by adding 0.4 ml of ice-cold assay buffer, and tubes were then centrifuged at 3000*g* for 15 min at 4°C. The pellet was washed twice and centrifuged as described above. The radioactivity of the pellet was counted. The specificity of IGF-I binding to its receptors was investigated using competitive inhibition studies with labeled IGF-I and unlabeled D-Trp^6-LH-RH (160 pmol–1.6 μM), SS-14 (150 pmol–1.5 μM), and porcine insulin (1–1000 ng).

LH serum levels were also determined by radioimmunoassay. The sensitivity of the standard curve was 13 pg and the intraassay variance was 3.6%.

Mathematical Analysis of the Binding Data. The counts of radioactivity obtained from filters or tubes containing membrane particles were corrected by deducting the background count from assays in which no membranes were added. The LIGAND-PC computerized curve fitting program of Munson and Rodbard (14) was used to determine the types of receptor binding, dissociation constant (K_d), and the maximal binding capacity of receptors (B_{max}). Statistical evaluation of data was performed by one-way analysis of variance and Duncan's new multiple range test and $P < 0.05$ was accepted as statistically significant.

Results

Treatment with microcapsules of D-Trp^6-LH-RH alone or in combination with somatostatin analog RC-160 significantly inhibited the growth of MXT mammary cancer. The tumor volume was markedly reduced at the end of treatment period in all of the groups as

compared with the controls (Table I). Analog RC-160, given alone, caused a significant reduction in tumor volume expressed as percent change (1490 ± 127%) when compared with controls (4315 ± 842%) ($P < 0.01$). D-Trp^6-LH-RH microcapsules resulted even in a greater decrease of percent change in tumor volume when given alone (398 ± 54%, $P < 0.01$) or in combination with RC-160 (316 ± 72%, $P < 0.01$). Ovariectomized mice showed the greatest decrease in percent change in tumor as compared with controls. However, there were no significant differences in the percent change in tumor volume between groups of animals treated with D-Trp^6-LH-RH, RC-160, or the combination. The greatest decrease in tumor weight was also obtained after ovariectomy (0.17 ± 0.08 g) as compared with control tumors (0.67 ± 0.12 g). The treatment with D-Trp^6-LH-RH alone or in combination with RC-160 caused significant reduction in tumor weight to 0.32 ± 0.10 g and 0.29 ± 0.09 g, respectively. RC-160 alone caused only moderate reduction to 0.38 ± 0.11 g. Other details of this study and pathologic results are described elsewhere (15).

Serum LH levels are shown in Table II. Serum LH level in the control group was 606 ± 185 pg/ml. After treatment with D-Trp^6-LH-RH alone or in combination with RC-160, the serum LH levels decreased to 250 ± 69.8 and 361 ± 70.6 pg/ml, respectively. Treatment with RC-160 did not influence serum LH level (612 ± 183 pg/ml). Surgical ovariectomy resulted in a highly significant increase in the serum LH level to 3076 ± 372.3 pg/ml.

Membrane Receptors for D-Trp^6-LH-RH. The optimal conditions for binding of ^{125}I-labeled D-Trp^6-LH-RH were found to be similar to those previously described (3). The characteristics of binding of D-Trp^6-LH-RH to the membrane receptors on the MXT cancer cells were determined using competitive inhibition studies. The displacement of labeled D-Trp^6-LH-RH (Fig. 1) and the Scatchard analysis of these data (Fig. 2) indicated that in membranes of MXT mammary cancers, labeled peptide was bound to the single class of noncooperative receptor sites ($K_d = 29.3 \times 10^{-9}$ M; $B_{max} = 4.55$ pmol/mg membrane protein). To determine whether D-Trp^6-LH-RH, SS-14, and IGF-I were bound to the same or different binding sites, we have also performed experiments in which the binding of

Table II. Serum LH Level in Mice Bearing MXT Mammary Tumors after Treatment with LH-RH and SS-14 Analogs[a]

Treatment	LH (pg/ml)
Vehicle (controls)	606.0 ± 185.0
D-Trp^6-LH-RH	250.0 ± 69.8
RC-160	612.0 ± 183.0
D-Trp^6-LH-RH + RC-160	361.0 ± 70.6
Ovariectomy	3076.0 ± 372.3[b]

[a] Results are mean ± SE. Statistics (versus control) were made using one-way analysis of variance.
[b] $P < 0.01$.

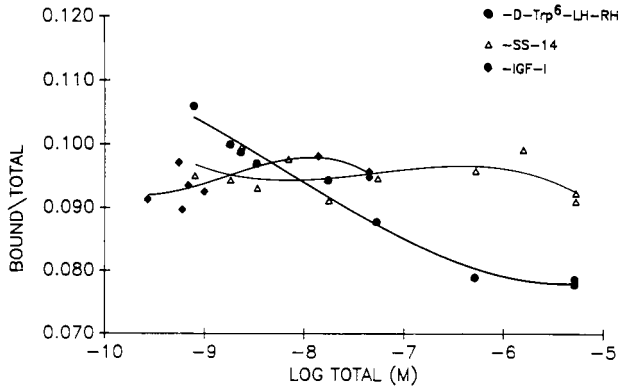

Figure 1. Displacement of ^{125}I,D-Trp^6-LH-RH (100–140 pmol) by D-Trp^6-LH-RH (160 pmol–1.6 μM), SS-14 (150 pmol–1.5 μM), and IGF-I (1–100 ng) in the control membranes of MXT mammary cancer cells. The individual data points are means of triplicate determinations. Percentage coefficient of variation was between 7 and 17%.

Table I. Effect of D-Trp^6-LH-RH and Somatostatin Analog RC-160 on Growth of the MXT Mammary Carcinoma in Mice[a]

Treatment	Tumor volume				Tumor weight	
	Initial (mm^3)	Final (mm^3)	% change from Day 0[b]	P^c		P^d
Vehicle (control)	88 ± 68.2	1975 ± 514	(+)[e] 4315 ± 842		0.67 ± 0.12	
D-Trp^6-LH-RH	88 ± 45.4	518 ± 155	(+) 398 ± 54	0.01	0.32 ± 0.10	0.05
RC-160	74 ± 32.4	1138 ± 342	(+) 1490 ± 127	0.01	0.38 ± 0.11	
Combination of D-Trp^6-LH-RH + RC-160	79 ± 27.2	498 ± 107	(+) 316 ± 72	0.01	0.29 ± 0.09	0.05
Ovariectomy	101 ± 52.8	15 ± 13	(−) 22 ± 62	0.01	0.17 ± 0.08	0.01

[a] Results are mean ± SE.
[b] Calculated on the basis of individual responses.
[c] Duncan's new multiple range test.
[d] Student's t test.
[e] +, increase; −, decrease.

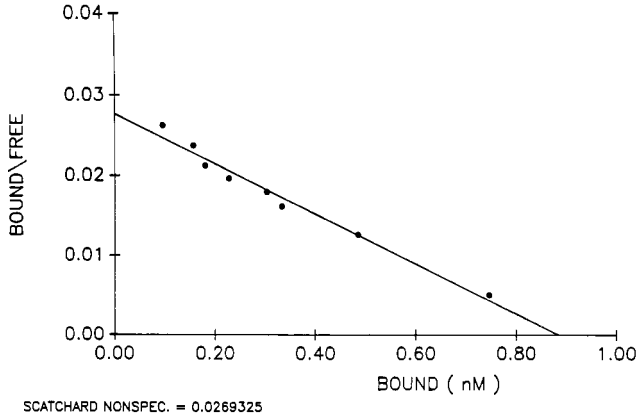

Figure 2. Scatchard plot of D-Trp^6-LH-RH binding to the control membranes of MXT mammary cancer cells. The individual data points are means of triplicate determinations.

labeled D-Trp^6-LH-RH was carried out in the presence of increasing amounts of SS-14 (150 pmol–1.5 μM) and IGF-I (0.1–100 ng). Both SS-14 and IGF-I were without effect (Fig. 1), indicating the specificity of our receptor assay for D-Trp^6-LH-RH.

Administration of microcapsules of D-Trp^6-LH-RH alone or in combination with RC-160 significantly decreased the binding capacity (0.96 ± 0.45 and 0.58 ± 0.14 pmol/mg membrane protein, respectively), and slightly increased the dissociation constant of D-Trp^6-LH-RH binding (42.3 ± 7.22 × 10^{-9} M and 38.1 ± 16.7 × 10^{-9} M, respectively) to the membrane binding sites on MXT cancer cells (Fig. 3). Treatment with RC-160 or ovariectomy did not significantly influence the characteristics of D-Trp^6-LH-RH binding to the MXT cancer membrane receptors (Fig. 3).

Membrane Receptors for SS-14. [Tyr^{11}]SS-14 binding to the membranes of MXT mammary cancer cells was specific and saturable. D-Trp^6-LH-RH (160 pmol–1.6 μM) and IGF-I (0.1–100 ng) were unable to displace ^{125}I-labeled [Tyr^{11}]SS-14 from the binding sites on MXT mammary cancer cells. Analysis of the displacement of labeled SS-14 by unlabeled SS-14 suggests that in control MXT cancer cells, labeled peptide was bound to only one class of membrane receptor sites (K_d = 7.51 × 10^{-9} M; B_{max} = 0.90 pmol/mg membrane protein) (Figs. 4 and 5).

In studies using control membranes, we have also obtained curvilinear Scatchard plot concave upwards (Hill coefficient less than 1.0) which can imply the heterogeneity of binding sites (two classes of binding sites). Using the F ratio test (14) we were unable to prove that a two-site model provides a statistically better fit than the one-site model. Therapy with D-Trp^6-LH-RH alone did not change binding characteristics of SS-14 receptors on MXT mammary cancer cells (Fig. 6). Chronic treatment with microcapsules of RC-160, alone or in combination with D-Trp^6-LH-RH, significantly increased both the dissociation binding constant (18.6 ± 3.54 × 10^{-9} M and 10.0 ± 0.70 × 10^{-9} M, respectively) and the binding capacity (13.98 ± 1.75 pmol/mg membrane protein and 21.0 ± 4.0 pmol/mg membrane protein, respectively) of receptors for SS-14

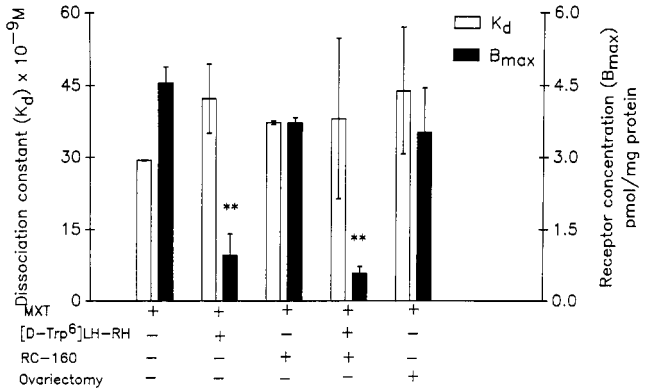

Figure 3. Characteristics of binding sites for D-Trp^6-LH-RH in the membranes of MXT mammary cancer cells after treatment with D-Trp^6-LH-RH and RC-160. Values shown are mean ± SE. Statistics (versus control) were made using Duncan's new multipoint range test. $^{**}P < 0.01$.

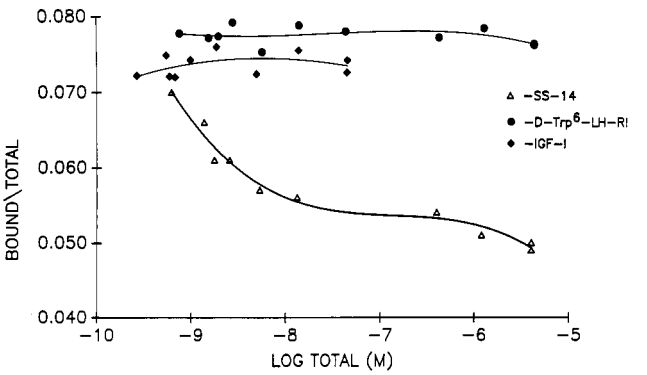

Figure 4. Displacement of [^{125}I,Tyr^{11}]SS-14 (90–120 pmol) by SS-14 (150 pmol–1.5 μM), D-Trp^6-LH-RH (160 pmol–1.6 μM), and IGF-I (1–100 ng) from the control membranes of MXT mammary cancer cells. The individual data points are means of triplicate determinations. Percentage coefficient of variations was between 7 and 17%.

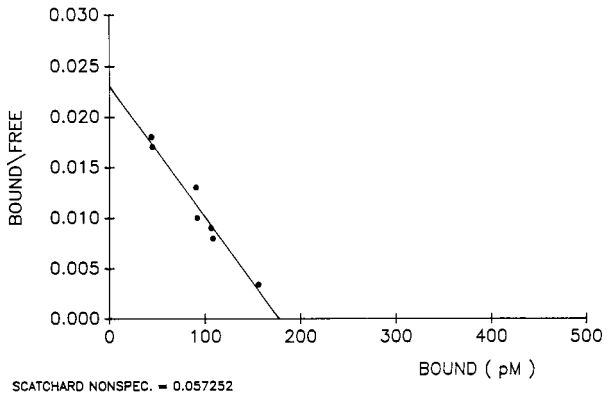

Figure 5. Scatchard plot of SS-14 binding to the control membranes from MXT mammary cancer cells. The individual data are means of triplicate determinations.

on MXT cancer cells. Ovariectomy did not significantly alter the dissociation binding constant or the binding capacity of receptors for SS-14.

Membrane Receptors for IGF-I. Specific binding sites for IGF-I were also found in the membrane fractions of control and treated MXT mammary cancer cells. The characteristics of IGF-I binding in MXT cancer cells from control animals are shown in Figures 7 and 8. The specific binding was reproducible and only one class of high affinity receptors was found. The affinity of binding was in the nanomolar range (K_d = 4.57 × 10^{-9} M) and the binding capacity was 2.24 pmol/mg membrane protein. To investigate specificity of IGF-I binding to membranes of MXT mammary cancer cells, we performed competitive inhibition studies with labeled IGF-I and unlabeled D-Trp^6-LH-RH (160 pmol–1.6 μM) and SS-14 (150 pmol–1.5 μM). Unlabeled D-Trp^6-LH-RH and SS-14 did not displace labeled IGF-I from its receptors. In addition, another study using labeled IGF-I and unlabeled porcine insulin (1–1000 ng) was performed in order to determine whether IGF-I and insulin bind to the same receptors. Porcine insulin used in a 10-fold higher concentration did not displace labeled IGF-I from its receptors. Chronic treatment with D-Trp^6-LH-RH and RC-160 alone significantly decreased dissociation constants of receptors for IGF-I in MXT mammary cancer cells (Fig. 9). The largest decrease of dissociation binding constant was seen in the group treated with the combination of D-Trp^6-LH-RH and RC-160 and in ovariectomized animals (0.73 ± 0.06 × 10^{-9} M and 0.39 ± 0.10 × 10^{-9} M, respectively). Treatment with D-Trp^6-LH-RH and RC-160 alone significantly increased B_{max}, while the combination of these peptides and ovariectomy slightly decreased B_{max} of IGF-I receptors on MXT cancer cells, but this decrease was not statistically significant.

Discussion

Breast carcinoma is a leading cause of cancer-related deaths among American women. Hormonal responsiveness of breast tumors appears to be mediated

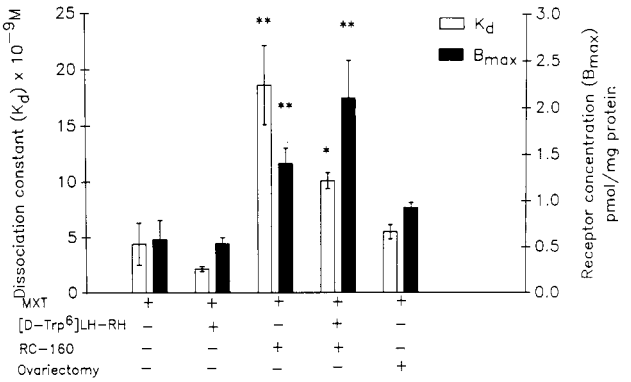

Figure 6. Characteristics of binding sites for SS-14 in the membranes of MXT mammary cancer cells after treatment with D-Trp^6-LH-RH and RC-160. Values shown are mean ± SE. Statistics (versus control) were made using Duncan's new multiple range test. *P < 0.05, **P < 0.01.

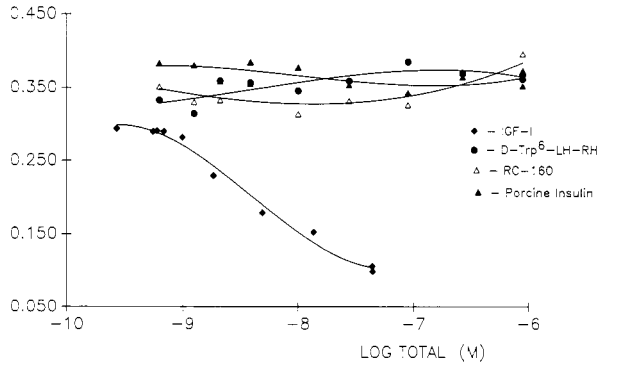

Figure 7. Displacement of ^{125}I-IGF-I (0.4–0.5 ng) by IGF-I (1–100 ng), D-Trp^6-LH-RH (160 mol–1.6 μM), SS-14 (150 pmol–1.5 μM), and porcine insulin (1–1000 ng) from the control membranes of MXT mammary cancer cells. The individual data points are means of triplicate determinations. Percentage coefficient of variations was between 6 and 12%.

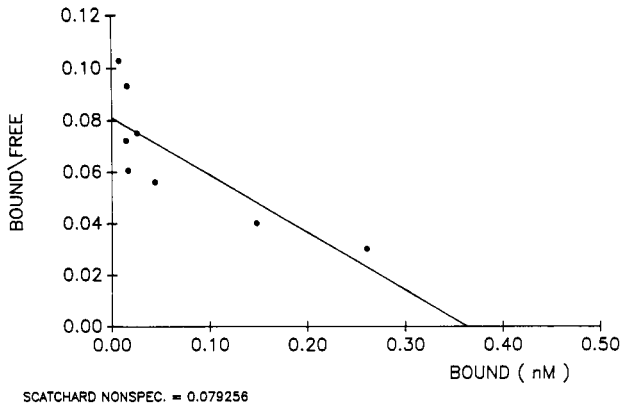

Figure 8. Scatchard plot of IGF-I binding to the control membranes of MXT mammary cancer cells. The individual data points are means of triplicate determinations.

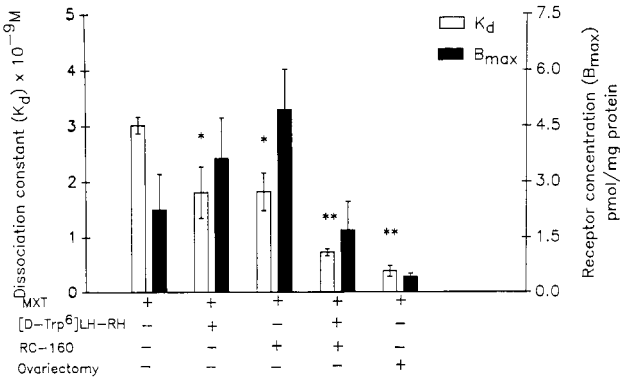

Figure 9. Characteristics of binding sites for IGF-I in the membranes of MXT mammary cancer cells after treatment with D-Trp^6-LH-RH and RC-160. Values shown are mean ± SE. Statistics (versus control) were made using Duncan's new multiple range test. *P < 0.05, **P < 0.01.

primarily by different hormone receptors. Watson et al. (16) have determined that the MXT tumor is estrogen dependent and estrogen receptor positive.

Previously it was reported that the mouse is resistant to the paradoxical inhibitory effects of agonistic analogs of LH-RH on the pituitary-gonadal axis (17). However, some paradoxical inhibition occurred after chronic daily administration of LH-RH agonists as shown by the reduction in ovarian weights and decrease in progesterone levels (1). Subsequently, we demonstrated that D-Trp-6-LH-RH administrated in the form of constant release microcapsules will strongly inhibit the pituitary-gonadal axis in female and male mice (18, 19). In our study chronic treatment with D-Trp^6-LH-RH alone or in combination with RC-160 in our experiments produced a decrease in ovarian and uterine weights and serum LH levels. Our results confirm that chronic treatment with LH-RH agonists, especially in delayed release formulation, suppresses the pituitary-gonadal axis. The reduction of tumor weight and volume after chronic treatment with D-Trp^6-LH-RH which was found in our experiments is thought to be mainly due to inhibition of pituitary and gonads (1). However, direct antitumor effects of LH-RH analogs on breast cancers are also possible since LH-RH receptors were found in different human mammary cancer cell lines and biopsy samples of human breast cancer (3, 20). Inhibitory effects *in vitro* on breast cancer lines (4) and the response of postmenopausal women with breast cancer to LH-RH agonists also suggests a direct action on mammary tumors.

LH-RH agonist D-Trp^6-LH-RH is a proper radioligand for LH-RH receptor studies (3, 21, 22). The sensitive multipoint micromethod we have used in our experiments was shown to be useful for determination of binding characteristics of receptors for D-Trp^6-LH-RH in human breast cancer samples (3), human and experimental prostate cancers (21), and normal tissues (21–23). In our study, homologous displacement assays showed that in MXT mammary cancers, D-Trp^6-LH-RH binds to one class of specific, saturable, noncooperative membrane-binding sites. Treatment with RC-160 alone, as well as ovariectomy, did not significantly change the binding properties of receptors for D-Trp^6-LH-RH on the membranes of MXT mammary cancers. Results of chronic treatment with RC-160 indicate that somatostatin analogs do not influence characteristics of MXT mammary membrane receptors for D-Trp^6-LH-RH in a manner of heterologous regulation. Down-regulation of LH-RH receptors after chronic continuous exposure to high concentrations of LH-RH or its analogs was demonstrated previously in pituitary cells (24, 25). Additional studies are necessary to determine whether the threshold for the initiation of changes in receptors' sensitivity differs between LH-RH binding sites on pituitary cells and those on mammary cancer cells.

In our experiment, chronic treatment with LH-RH agonist D-Trp^6-LH-RH (which is 15–100 times more potent than LH-RH) (6) produced down-regulation in receptors for D-Trp^6-LH-RH in MXT cancer cells, without significant changes in receptor affinity. The same pattern of receptor changes was seen in androgen receptors in the prostate after endocrine manipulations (11, 26). Down-regulation of LH-RH receptors is believed to involve physical internalization of agonist-occupied receptors, strongly suggesting a direct effect of LH-RH and its agonists on target cells. All of these findings and the results of our study support the view that D-Trp^6-LH-RH in addition to its principal effects, mediated through the suppression of the pituitary-gonadal axis, may exert some effects on growth of MXT cancer cells also by a direct action.

This study demonstrates the existence of somatostatin receptors in membrane fractions of MXT breast cancer cells. High affinity somatostatin receptors were found in membrane fractions of Dunning prostate tumors and normal rat prostate (21), in normal human pancreas, human pancreatic cancer, MIA PaCa-2 pancreatic cancer cell line (22), rat pituitary cells (27), pituitary and brain tumors (28, 29) as well as in human breast cancer (3, 28).

Our experiments showed that somatostatin receptors on MXT cancer cells are saturable and specific. D-Trp^6-LH-RH and IGF-I were unable to displace labeled somatostatin from its binding sites on MXT cancer cells. It seems that somatostatin binds to only one class of binding sites on membranes of MXT cancer cells. The appearance of curvilinear Scatchard plot in our studies with samples from control animals bearing MXT cancer could be due to heterogeneity of binding sites (or ligand), negatively cooperative site-site interactions, or to a two-step/three-component binding reaction (30). *F* ratio test (14) did not prove that a two-site model provides a statistically significantly better fit than the one-site model. In all of our studies we have used the same batch of unlabeled peptide and the labeled peptide which was iodinated in one single experiment. Therefore, it seems unlikely that curvilinear Scatchard plot is due to the heterogeneity of the ligand used in our studies. Two-step/three-component reactions with ternary complex formation usually occurs in adenylate cyclase-coupled receptor systems. In the transduction of somatostatin-induced intracellular signal, the inhibition of adenylate cyclase represents one of the most important steps. Consequently, it seems that the curvilinear Scatchard plot in our experiments was due to a two-step reaction involving adenylate cyclase as a third component.

RC-160, a potent somatostatin agonist (31), was successfully used as an adjunct to LH-RH agonists in the treatment of experimental prostate cancer (32) as well as experimental pancreatic cancer (22). Somatostatin and its analogs in addition to suppression of

growth hormone and prolactin levels (5, 31) appear to inhibit directly the action of endogenous growth factors, such as epidermal growth factor (EGF), by mechanisms involving interference with signal transmission. Prolactin may be a cofactor in breast cancer and GH promotes various growth factors that may be involved in the proliferation and transformation of malignant cells (8). Therefore, somatostatin agonists might inhibit breast cancers by interfering with the secretion of GH and prolactin and with the action of endogenous growth factors.

In our experiments, RC-160 alone or in combination with D-Trp^6-LH-RH significantly increased both K_d and B_{max} of receptors for somatostatin in MXT cancer cells. Homologous up-regulation of somatostatin receptors after prolonged treatment with somatostatin agonists was also found in studies with experimental pancreatic cancer (22). After interaction with its receptor, somatostatin is internalized by a process which is time, temperature, and ligand concentration dependent (33). Significant proportions of receptors may be shuttled back to the cell surface through the process of receptor recycling, which has been also described for insulin and transferrin receptors (34, 35). In addition, a prolonged decline of membrane surface receptors produced by internalization can lead to *de novo* synthesis of somatostatin receptors. Together, both recycling and *de novo* synthesis could contribute to the increase in number of somatostatin receptors after prolonged treatment with somatostatin agonist RC-160.

Heterologous regulation of SS-14 receptors in different tissues also must be considered. In rat pituitary cell cultures, Schonbrunn and Tashjian (27) found competition of ACTH and glucagon with somatostatin for binding to cytosol. Moyse et al. (36) suggested a heterologous regulation of somatostatin receptors on human pituitary adenomas by GH.

Taken together, the presence of somatostatin membrane receptors on MXT mammary cancer cells, changes of their characteristics after chronic treatment with RC-160 as well as inhibitory action *in vitro* of RC-160 on breast cancer lines suggest that, in addition to the principal effect of somatostatin analogs on GH, prolactin, and growth factors, a direct effect on MXT cancer cells could be involved. Our results support the suggestions made by others (22) about a direct effect of somatostatin analogs on tumor cells.

IGF-I acts as a growth promoter on several mammalian cells (37). There are at least two mechanisms by which the presence of IGF-I receptors might influence the proliferation of tumors. First, it is possible that IGF-I receptor-positive cancers may exhibit a dependence on exogenous IGF-I for optimum proliferation, in a manner analogous to estrogen dependence of estrogen receptor-positive breast cancers (10). Second, in view of *in vitro* data showing the constitutive production of IGF-I by estrogen receptor-negative breast cancer cells, it is possible that neoplasms that have IGF-I receptors produce IGF-I in an unregulated manner (8, 10). Therefore, IGF-I may be a hormonally regulated autocrine growth stimulator (38).

In the specimens of MXT mammary cancer, we have found a substantial number of IGF-I receptors. Membrane IGF-I receptors on MXT cancer cells are specific, since D-Trp^6-LH-RH and SS-14 did not displace labeled IGF-I from its binding sites. Insulin, also, did not show an affinity for IGF-I receptors on MXT cancer cells. The same pattern was found using membranes of rat liver cells (39).

In our studies on MXT mammary cancer cells, IGF-I was bound to the one class of saturable and noncooperative binding sites. Characteristics of IGF-I membrane receptors on MXT cancer cells ($K_d = 3.01 \pm 0.15 \times 10^{-9}$ M; $B_{max} = 2.24 \pm 0.96$ pmol/mg membrane protein) are in the same range as IGF-I receptors on different human breast cancer cells (9, 10). Dissociation constant of IGF-I receptors was changed significantly after chronic treatment with D-Trp^6-LH-RH, RC-160, a combination of these peptides, and after ovariectomy. A direct effect of LH-RH and SS-14 analogs on the affinity of IGF-I receptors cannot be excluded, since heteroregulation of the receptors for different growth factors was previously reported (40). The presence of IGF-I membrane receptors on the membranes of MXT mammary cancer cells implicates IGF-I in the growth of mammary cancers, as it was previously proposed (9, 10). The importance of IGF-I for the growth of mammary cancers opens the possibility of developing competitive antagonists of IGF-I receptors or anti-IGF-I receptor-blocking antibodies as a new therapeutic approach of the mammary cancers, as suggested by Pollak *et al.* (10).

Together, analyses of characteristics of membrane receptors for D-Trp^6-LH-RH, SS-14, and IGF-I on MXT mammary cancer cells *in vitro* effects and tumor regression in response to treatment with analogs support the view that LH-RH, SS-14, and IGF-I play an important role in the regulation of growth of mammary cancers. Changes in receptor binding seen after treatment with D-Trp^6-LH-RH and RC-160 could be caused in part by the direct effect of these analogs on MXT cancer-binding sites. Down-regulation of receptors for D-Trp^6-LH-RH on the membranes of MXT cancer cells along with a slight increase of K_d in our experiments implies a decrease in direct effect of D-Trp^6-LH-RH on MXT cancer cell growth after prolonged treatment with D-Trp^6-LH-RH. Therefore, it seems probable that the reduction of tumor weight and volume during treatment with D-Trp^6-LH-RH alone or in combination with RC-160 is due mainly to estrogen deprivation and inhibition of GH and prolactin. An increase of the number of SS-14 receptors after treatment with RC-160 could be of importance for the treatment of breast cancers. Direct effect of somatostatin analogs on tumor

growth might become more intense during a prolonged treatment with these drugs. Our results and previous findings (3) suggest the merit of continued therapeutic trials with the combination of LH-RH agonists or antagonists and somatostatin analogs in the management of breast carcinoma.

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