Gonadotropin-Releasing Hormone Binding Sites in Human Breast Carcinoma

Abstract. *Gonadotropin-releasing hormone analogs can cause regression of hormone-dependent breast carcinomas. These effects are thought to be mediated through the inhibition of gonadotropic and steroid hormones. These analogs may also act directly on the tumor because they are effective in treating breast cancer in some postmenopausal women. The presence of specific binding sites for gonadotropin-releasing hormone was demonstrated in human breast carcinomas by means of a novel approach of ligand immunoblotting. The results indicate a possible mechanism by which the peptide has direct effects on this tissue. These binding proteins were not detectable in non-neoplastic breast tissue.*

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Gonadotropin-releasing hormone (GnRH) and its analogs have recently been used in the therapy of gonadal hormone–sensitive cancers. Prostatic tumors in humans have regressed after treatment with GnRH analogs (*1*), and similar effects have been demonstrated experimentally in rats (*2*). Growth of hormone-sensitive mammary tumors in rats is inhibited by GnRH agonists and antagonists (*3*), and clinical studies have indicated beneficial effects of GnRH analogs in the treatment of metastatic breast cancer (*4*). The major mechanism of action of GnRH inhibition of tumor growth is thought to be a desensitization of the pituitary to GnRH with a consequent decline in gonadotropin secretion and gonadal hormone production. There is, however, an indication that GnRH exercises its effects on certain breast tumors by mechanisms independent of gonadal suppression because some postmenopausal women, in whom gonadal steroids are lacking, have responded to GnRH (*5*). We have considered the possibility that GnRH has direct effects on breast carcinomas and have demonstrated the presence of specific binding sites for GnRH in a high percentage of ductal breast carcinomas. These binding sites are rare in lobular breast carcinomas and absent from non-neoplastic breast tissue. A ligand immunoblotting technique established that the binding protein in breast carcinomas has a molecular weight of 64,000, which is identical to that for the human pituitary GnRH receptor (*6*).

Primary human breast carcinomas removed at surgery were processed as described (legend to Fig. 1). Solubilized membrane proteins were electrophoretically separated and transferred to nitrocellulose by Western blotting, and the GnRH binding protein was visualized by a ligand-antibody-peroxidase technique (Fig. 1). A distinct band of approximately 64,000 molecular weight was present in blots of certain breast carcinomas and of a human intraductal breast carcinoma cell line (Fig. 1A). The band was absent from blots of normal breast tissue and tissue showing fibroadenosis (Fig. 1C; Table 1). The GnRH binding protein in breast carcinomas was indistinguishable in its electrophoretic migration from that of the human pituitary GnRH receptor (Fig. 1A). The rat pituitary GnRH receptor was also visualized by this method and found to have a molecular weight of 60,000, as has been reported (*7*) (Fig. 1A). Specificity of the method was indicated by the disappearance of the staining reaction when one or another of the reagents was omitted. Furthermore, no staining was evident when nonimmune rabbit serum or an antiserum to GnRH (which binds to the COOH and NH_2 termini of the molecule) were used (Fig. 1B). The COOH- and possibly the NH_2-terminal sequences of GnRH are thought to interact with the receptor and thus would not be available for binding by the

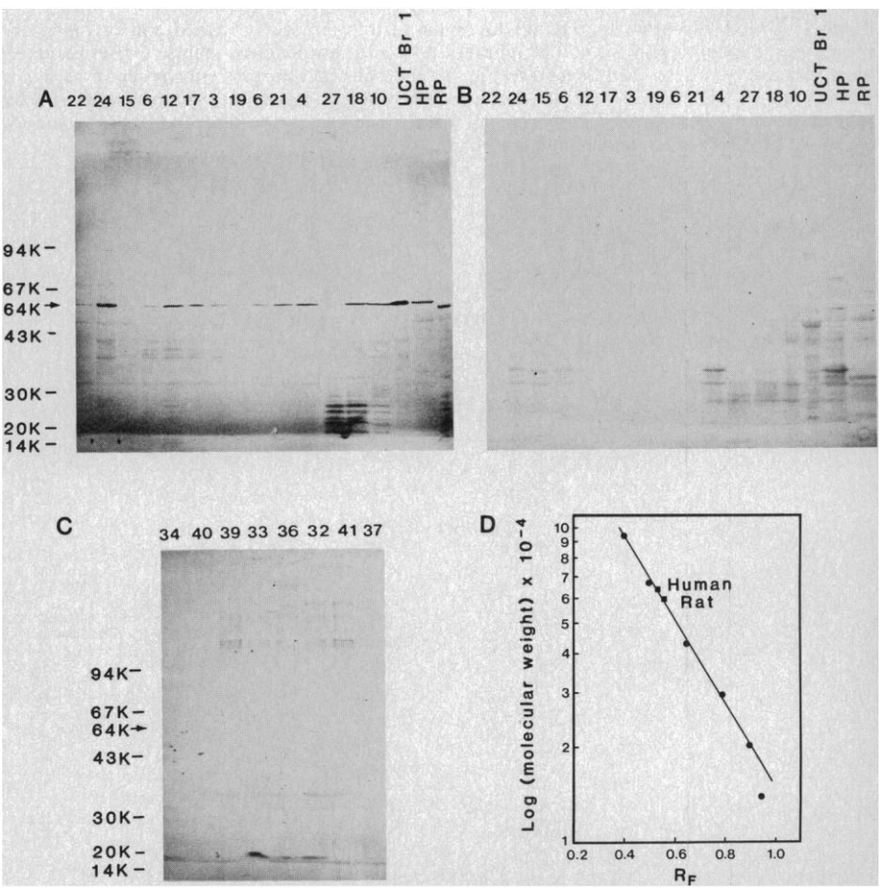

Fig. 1. (A to C) Ligand immunoblot analysis of solubilized breast tumor membranes after sodium dodecyl sulfate electrophoresis and Western blotting. (A and C) Incubation with antiserum 1076; (B) incubation with antiserum 432. The patient specimens are identified as described in Table 1; also included are human breast tumor cell line (UCT Br 1) (*18*), human pituitary (HP) (obtained at autopsy) (*6*), and rat pituitary (RP). Membranes were prepared from tissues and solubilized as described (*19*). Solubilized membrane preparations were subjected to ligand immunoblotting as described (*20*). Non-neoplastic breast tissue included tissue showing fibroadenosis and material obtained at mammaplasty. Apparently normal tissue surrounding the tumors was not included in the study because of the possible presence of scanty tumor tissue. (D) Log (molecular weight) plotted against relative migration (R_F) of low molecular weight markers (Pharmacia) and receptor bands.

antiserum (*8*). Only antiserum 1076, which is directed toward the middle region of GnRH (*9*), visualized the GnRH receptors.

The presence of specific GnRH binding sites in breast carcinoma tissues was confirmed by means of a different technique. Instead of using an antiserum to detect GnRH bound to nitrocellulose-immobilized proteins, we conducted conventional binding studies using the same membrane preparation and an ^{125}I-labeled GnRH analog ([^{125}I]GnRH A) (Fig. 2). Increasing doses of unlabeled GnRH displaced the labeled analog, with a median effective dose (ED_{50}) of approximately $2 \times 10^{-8}M$. The affinity of GnRH binding to breast carcinoma binding sites is therefore apparently lower than that of binding to the human pituitary GnRH receptor (ED_{50}, $5.3 \times 10^{-9}M$; binding affinity, $2.1 \times 10^{8}M$) (*6*). The affinity of GnRH binding to human corpus luteum is also low (*10*). Since rat ovarian granulosa cells and rat pituitary receptors have a similar high affinity for GnRH (*11*), it is unclear whether these are species- or tissue-related differences. Specificity of binding was indicated by an inability of the biologically inactive GnRH fragment (1–5) ($10^{-5}M$) and thyrotropin-releasing hormone (TRH, $10^{-5}M$) to displace appreciable amounts of [^{125}I]GnRH A. In those instances when adequate mammary tissue was available, we compared the results of specific [^{125}I]GnRH A binding to those obtained by visualization on immunoblotting. Specific [^{125}I]GnRH A binding greater than 300 count/min ($P < 0.05$, unpaired t test) was considered positive. Seven breast specimens that were positive for [^{125}I]GnRH A binding were also positive by the immunoblotting technique, whereas four specimens were negative in both systems. One specimen, which was not positive for [^{125}I]GnRH A binding, showed low positive staining on nitrocellulose. The immunoblotting technique appears to be more sensitive and specific, and exhibits a lower background, than the conventional [^{125}I]GnRH A binding technique, which shows relatively high nonspecific binding.

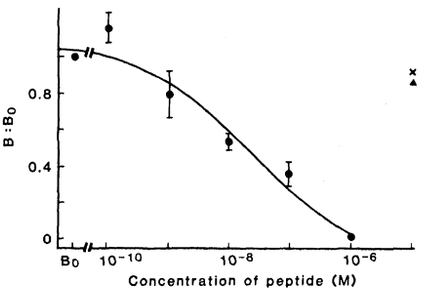

Fig. 2. Displacement of [^{125}I]GnRH A ([D-Ala^6,$N^{\alpha\text{-methyl}}$ Leu^7,Pro^9-N$^{\text{ethylamide}}$]-GnRH) bound to human ductal breast tumor membranes by GnRH (Bachem) (●), TRH (Serva) (▲), and GnRH (1–5) (X). Binding assays with membranes prepared as described (legend of Fig. 1) were carried out as described (*21*). In brief, the membrane pellet was resuspended in 10 m*M* tris-HCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, and 0.1 percent bovine serum albumin at *p*H 7.4 to a final concentration of 10 mg/ml (4 mg per tube). The solution was incubated with [^{125}I]GnRH A (1400 μCi/μg) and increasing concentrations of unlabeled peptide for 60 minutes at 4°C. Incubation was terminated by addition of 0.04*M* sodium phosphate, 0.15*M* NaCl, and 1.0 percent BSA at *p*H 7.4 and subsequent filtration through glass fiber filters (GF/C, Whatman). After several washings, membrane-bound label retained on the filters was measured in a gamma counter. Dose-response curves were analysed by a computerized fitting program (ALLFIT) with a four-parameter logistic function (*22*). Values show the mean ± standard error of the mean of triplicate incubations. The ratio B:B_0 represents the amount of [^{125}I]GnRH A remaining after displacement compared to the amount initially bound.

Table 1. GnRH receptors in relation to tumor histological classification and estrogen and progesterone receptors in women of different ages and reproductive status. GnRH receptors were visualized as described (legend to Fig. 1). Estrogen and progesterone receptor assays of breast tumors were carried out as described (*23*). All non-neoplastic tumors studied from premenopausal (seven) and postmenopausal (four) subjects were negative for GnRH receptors. Symbols: N.D., not detected; +, distinct band; low +, faint band; −, no band.

Carcinoma type	Patient		Receptors (fmol/mg) for:		Band for GnRH receptor
	Number	Age (years)	Estrogen	Progesterone	
Ductal			*Premenopausal*		
	1	40	129	285	+
	2	42	N.D.	52	+
	3	42	N.D.	N.D.	Low +
	4	44	33	N.D.	+
	5	45	N.D.	N.D.	+
	6	48	11	N.D.	Low +
	7	49	277	N.D.	+
	8*	54	N.D.	N.D.	−
			Postmenopausal		
	9	60	20	N.D.	−
	10	60	N.D.	N.D.	+
	11	61	49	N.D.	+
	12	63	44	71	+
	13	64	N.D.	N.D.	+
	14	67	24	68	+
	15	70	327	101	Low +
	16	71	79	28	+
	17	71	10	N.D.	+
	18	73	235	301	+
	19	77	294	N.D.	−
	20	82	630	645	+
	21	85	N.D.	N.D.	+
Lobular			*Premenopausal*		
	22	30	40	67	Low +
	23	49	N.D.	N.D.	−
	24†	51	129	N.D.	+
			Postmenopausal		
	25	57	185	178	−
	26	68	N.D.	N.D.	−
	27	74	450	399	−
	28	77	N.D.	N.D.	−
	29	91	24	145	−
	30	92	N.D.	N.D.	−

*Exact stage of menopause not known. †Perimenopausal.

Comparisons of clinical and biochemical data show that the presence of GnRH binding sites is not related to the age and reproductive status of the patient or the presence of estrogen or progesterone receptors in the tumor tissues. However, GnRH binding was prevalent in ductal carcinomas (19 of 21) compared to the lobular carcinomas (2 of 9) (Table 1).

Seppala and Wahlstrom (*12*) have detected GnRH immunoreactivity in mammary ductal carcinomas using an antiserum similar to the one used here. It is possible, therefore, that they identified occupied GnRH binding sites in the mammary tumor cells. The observation that GnRH is present in human breast milk (*13*) suggests that there may be cells producing GnRH as well as cells with receptors for the peptide in the lactating

mammary gland. The absence of GnRH binding sites in non-neoplastic tissues suggests that carcinogenic transformation of normal breast cells is in some instances accompanied by the appearance of membrane proteins not normally present, or present only in undetectable amounts, in the nonlactating gland.

Evidence that the GnRH binding sites in tumor tissue are GnRH receptors may be provided by demonstrating that GnRH can affect cellular function. GnRH agonists have been reported to inhibit the estrogen-dependent growth of cultured mouse mammary tumor cells in a dose-dependent fashion (*14*), and inhibition of the growth of a single human breast cancer cell line in culture by GnRH analogs has been described (*15*). GnRH analogs also inhibit the incorporation of [^{3}H]thymidine and [^{14}C]leucine into MCF-7, MDA-MB-231, and ZR-75-1 human breast tumor cells in culture (*16*). These ductal cell lines were all positive for GnRH binding sites.

Although a major mechanism of inhibition of breast carcinomas by GnRH analogs is likely to be the inhibition of gonadotropin secretion and decline in gonadal steroid hormone production, the efficacy of the hormone in the treatment of some postmenopausal women with breast cancer and the effects demonstrated in vitro argue in favor of a direct effect of GnRH analogs on breast tumor cells. Our demonstration of specific GnRH binding sites in certain breast carcinomas illustrates a potential mechanism for these effects. A precedent for GnRH binding sites in a steroid hormone–sensitive tissue has been shown in rat prostatic tumors (*17*). The significance of GnRH binding sites in human breast carcinomas and their relevance in clinical management of breast cancer remain to be determined. The possibility of a correlation between the direct effects of GnRH on breast carcinoma cell lines and the presence of GnRH binding sites in the cell lines merit further study.

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19. Tumors and normal breast tissue were excised, rapidly frozen in liquid nitrogen, and stored at −70°C. The frozen tissue was diced and then homogenized in buffer containing 20 m*M* tris-HCl, 150 m*M* NaCl, 1 m*M* $CaCl_2$, and 1 m*M* phenylmethylsulfonyl fluoride at *p*H 8.0 on ice. Subsequent work was carried out at 0° to 4°C. The homogenate was centrifuged at 800*g* for 10 minutes, and the supernatant was centrifuged again at 800*g* for 5 minutes. The resulting supernatant was centrifuged at 10,000*g* or at 100,000*g* for 60 minutes. Membrane pellets were weighed and suspended in 125 m*M* tris-maleate, 160 m*M* NaCl, and 40 m*M* β-D-octylglucopyranoside (*p*H 6.0) to a final concentration of 100 mg/ml; the mixture was incubated at 0°C for 10 minutes and centrifuged at 10,000*g* for 15 minutes to remove nonsolubilized material. Membranes from tumors 27 and 18, which were prepared by centrifugation at 100,000*g*, exhibited low molecular weight bands (presumably nonspecific) that were not present in tumor membranes prepared by centrifugation at 10,000*g*.
20. Ligand immunoblotting was performed essentially as described [K. A. Eidne, D. T. Hendricks, R. P. Millar, *Endocrinology* **116**, 1792 (1985); this technique was based on the principle described by T. O. Daniel *et al.*, *J. Biol. Chem.* **258**, 4606 (1983)]. Protein (120 μg per lane) was loaded onto a 6 to 12 percent linear gradient polyacrylamide gel containing 0.1 percent sodium dodecyl sulfate; the protein was subjected to electrophoresis at 4°C under nonreducing conditions at 25 mA for 45 minutes and then at 35 mA for 60 minutes. The protein was then electrophoretically transferred to nitrocellulose in 50 m*M* tris, 200 m*M* glycine, and 20 percent (by volume) ethanol at 195 mA for 1 hour at 23° to 25°C. Protein molecular weight markers were stained with amido black, and the nitrocellulose strips with separated membrane proteins were incubated at 4°C overnight in buffer A [50 m*M* tris-HCl, 90 m*M* NaCl, and 50 mg of bovine serum albumin per milliliter (*p*H 8.0)] containing 10 percent fetal bovine serum. The nitrocellulose was then incubated in 10^{-6} *M* GnRH in buffer A for 45 minutes and washed eight times in buffer B [50 m*M* tris-HCl, 90 m*M* NaCl, and 5 mg of bovine serum albumin per milliliter (*p*H 8.0)], with subsequent incubation (45 minutes) in 10 ml of buffer A containing 200 μl of either antiserum 1076 (directed toward the middle region of GnRH) or antiserum 432 (directed toward the COOH and NH_2 termini of GnRH) or nonimmune rabbit serum. After the nitrocellulose was washed as before, incubations were carried out in 10 ml of buffer A containing 25 μl of affinity-purified horseradish peroxidase–conjugated sheep antibody to rabbit immunoglobulin G (Bio-Rad). The GnRH binding proteins were visualized by incubating them for 5 minutes with 4-chloro-1-naphthol [Sigma; 0.5 mg/ml in 50 m*M* tris-HCl, 150 m*M* NaCl (*p*H 8.0), and 0.0003 percent H_2O_2]. The reaction was terminated by several washes in distilled water.
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24. We thank J. Rivier for GnRH A; R. Milton for GnRH (1–5); R. J. Aitken for human breast tumor tissue; E. Dowdle, L. Wilson, and R. Prescott for steroid receptor assays and the UCT Br 1 cells; the Breast Clinic for patient data; and L. Odes, S. Benatar, and M. Berman for technical support. Supported by grants from the Medical Research Council, the University of Cape Town, and the National Cancer Association.

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Tissue Factor Gene Localized to Human Chromosome 1 (1pter → 1p21)

Abstract. *Tissue factor (tissue thromboplastin, coagulation factor III), a protein component of cell membranes, is an essential cofactor for factor VII–dependent initiation of blood coagulation. Since no tissue factor–deficient condition has been described, it is one of only a few proteins of the coagulation system for which the pattern of inheritance has not been ascertained. Because of the species–specificity of tissue factor activity and the availability of a very sensitive chromogenic assay, it was possible in the present study to use somatic cell hybrids to assign the chromosomal location of the tissue factor structural gene* (F3) *to human chromosome 1 (1pter → 1p21).*

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Tissue factor is a glycoprotein present in the membranes of most cells and is traditionally assigned to the extrinsic pathway of blood coagulation (*1*, *2*). Also known as tissue thromboplastin or coagulation factor III, tissue factor serves as a lipid-dependent cofactor for factor VII–mediated activation of factor X (*3–6*) and factor IX (*7*). That it may be the primary physiological initiator of blood coagulation (*1*) is supported by the observation that tissue factor is the only known protein in the pathway leading to blood coagulation for which a congenital deficiency has not been reported (*8*, *9*). Tissue factor regulation, both in terms of expression of activity at the cell membrane and at the genetic level, is impor-