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Failure to Detect Gonadotrophin-releasing Hormone Receptors in Human Benign and Malignant Breast Tissue and in MCF-7 and MDA-MB-231 Cancer Cells

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We have measured the binding of radiolabelled analogues of gonadotrophin-releasing hormone (GnRH) to homogenates of human breast cancer and benign breast tissue, and to MCF-7 and MDA-MB-231 cell lines. Although incubation of breast cancer homogenates with the ^{125}I-labelled GnRH agonist analogues, buserelin [(D-Ser tBU^{6})GnRH 1-9 ethylamide] and tryptorelin [(D-Trp^{6}) GnRH 1-9 ethylamide] appeared to show significant though low, specific GnRH agonist binding in a high proportion of breast cancers (32/42 for buserelin; 15/32 for tryptorelin) and benign breast tissues (13/16 for buserelin; 10/12 for tryptorelin), after correction for displaceable binding in control assay tubes, GnRH agonist binding to breast tissue was no longer apparent. The lack of specific binding was not due to inactivation of GnRH agonist tracers, as >86% of the unbound tracer was still capable of rebinding to fresh placental membranes after incubation with breast cancer homogenates. GnRH agonist did not bind to MCF-7 and MDA-MB-231 cells, however GnRH agonist tracer inactivation following exposure to these cells was very high. We have shown recently that human placental receptors bound salmon GnRH and chicken GnRH II as well as GnRH agonists, but not other isoforms of GnRH. However, no isoform of GnRH bound significantly to human breast tumour tissue.

In summary, we could not confirm the presence of specific GnRH binding sites in homogenates and membranes from human breast tissues in this study. Low levels of apparently specific binding of GnRH agonist tracers could be accounted for entirely by displacement of tracer from assay tubes. Inability to demonstrate specific binding was not due to extensive inactivation of GnRH tracers (although this may be a factor in the failure to demonstrate GnRH binding to MCF-7 and MDA-MB-231 cell lines).
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INTRODUCTION

CHRONIC TREATMENT of premenopausal breast cancer patients with gonadotrophin-releasing hormone (GnRH) agonist analogues causes desensitisation and down-regulation of the pituitary gland followed by profound suppression of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. This leads in turn to suppression of gonadal steroid secretion (medical gonadectomy) and to the inhibition of the growth of steroid receptor-positive breast tumours [1–3]. In postmenopausal women, however, inhibitory effects of GnRH analogues on tumour growth have also been observed [4], suggesting direct effects of GnRH analogues on breast cancer cell growth. Although circulating androgen levels may fall on treatment of post-menopausal women with GnRH analogues [5], tumour response may also be due to direct effects on breast tumour cell growth [6–8] and specific binding sites for GnRH analogues have been described in breast tumour cells [6, 7, 9–11]. We have shown that GnRH binding sites in human extrapituitary tissues differ from pituitary receptors in their affinity and specificity for GnRH and GnRH analogues [12–14]. These studies have now been extended to human breast tumour tissues and human breast cancer cell lines.

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MATERIALS AND METHODS

Materials

Sephadex G25 (fine) was obtained from Pharmacia (Milton Keynes, UK) and from Sigma Chemical Co. (Poole, UK). Sephadex G25 QAE-A was from Pharmacia. All other fine chemicals and reagents were from Sigma or from BDH (Poole, UK). Radiolabelled sodium iodide (Na^{125}I) was from Amersham International (Bucks, UK).

Chicken GnRH I and II were purchased from Peninsula Laboratories (Belmont, California, USA) and m GnRH from Ayerst Laboratories (Andover, Hants, UK). Salmon and lamprey GnRH were generous gifts of Dr J. King (MRC Regulatory Peptides Research Unit, University of Cape Town, RSA), and the GnRH agonist, buserelin [(D-Ser tBu^{6}) 1-9 GnRH ethylamide], was the kind gift of Dr J. Sandow (Hoescht AG, Frankfurt, Germany). All other GnRH analogues were purchased from Sigma.

Tracer preparation

Peptides were radioiodinated using the glucose oxidase/lactoperoxidase method [15], and were purified by chromatography on Sephadex G25 columns (1 × 40 or 1 × 60 cm) in 0.01 mol/l acetic acid–0.1% bovine serum albumin (BSA). Specific activity

of binding was calculated for each GnRH isoform or analogue tracer preparation as described previously [14], and ranged from 2.22 to 66.12 TBq/μg.

Tissues

Human breast cancers were recovered at operation for mastectomy. All tumours were shown to be histologically malignant (Table 1; patients 1–13). Benign tissue was a mixture of histologically non-malignant material from patients presenting with benign breast conditions, or tissues removed from peripheral regions of the breast distal to single small breast cancers (Table 1; patients 14–23). All material was immediately frozen and stored in liquid nitrogen until assayed for GnRH binding. Other tissue pieces were stored for steroid receptor measurements. On the day of the experiment, tissues were thawed, weighed and homogenised (Polytron homogenizer, Kinematica, Sweden) in ice-cold 0.3 mol/l sucrose–10 mmol/l Tris–HCl–1 mmol/l EDTA, pH 7.4 (SET medium; 10 ml/g) with two 10-sec bursts at full power. Homogenates were filtered through cheesecloth and aliquots assayed immediately for GnRH binding. In a few experiments, tumour membranes were prepared by centrifugation of homogenates at 1000 *g* for 10 min, followed by recentrifugation of the supernatant at 100 000 *g* for 60 min. The membrane pellet was resuspended in SET and stored at −20°C until required.

Table 1. Histological characterisation of some of the human breast material used for GnRH binding studies

Patient	Histology of tissue	$GnRH_A$ binding (% total counts added) Bt	NSB	(Bt–NSB)
1	Intraductual carcinoma	8.56	7.44	1.12
2	Invasive moderately differentiated carcinoma	9.54	7.80	1.74
3	Widespread intraductal carcinoma	9.14	7.49	1.64
4	Invasive lobular carcinoma	7.52	6.60	0.92
5	Adenocarcinoma	8.19	7.05	1.14
6	Invasive ductal carcinoma	8.69	7.02	1.67
7	Invasive carcinoma of no special type	9.14	8.71	0.43
8	Invasive ductal carcinoma of no special type	9.54	7.79	1.74
9	Invasive carcinoma of no special type	9.39	7.16	2.23
10	Ductal intraductal and infiltrative carcinoma	6.97	5.86	1.11
11	Invasive and intraductal carcinoma	9.22	7.82	1.40
12	Invasive carcinoma of no special type	8.73	7.57	1.16
13	Adenocarcinoma of ductal origin	8.13	6.76	1.37
14	Fibroadenoma	9.35	7.12	2.23
15	Fibroadenoma	10.96	8.16	2.79
16	Fibroadenoma	9.94	7.96	1.98
17	Fibroadenoma	8.49	6.98	1.50
18	Reduction mammoplasty	8.33	7.09	1.24
19	Necrotic hyperplasia (not cancer)	8.28	6.99	1.29
20	Breast fat distal to cancer	10.96	8.92	2.03
21	Breast fat distal to cancer	9.54	7.75	1.79
22	Breast fat distal to cancer	7.84	7.17	0.67
23	Breast fat distal to cancer	10.12	7.84	2.28
	Rat pituitary	20.98	6.90	14.08
	Control (no homogenate)	9.03	6.74	2.29

Samples from patients 1–13 were classified as breast cancers; samples 14–23 were classified as benign breast tissues. Tissue was homogenised at a concentration of 10 mg/ml and 100 μl aliquots incubated at 4°C for 2 h with radiolabelled buserelin (100 000 cpm per ml) with or without unlabelled buserelin (10 μg/tube). Although breast tissues showed a difference between binding in the absence and presence of cold agonist of 0.4–2.8%, controls with no homogenate showed a displacement of 2.29%. After correction for this difference, statistical significance of GnRH binding by breast tissues disappeared. However, rat pituitary controls consistently showed specific binding (14.08%). Bt = Total binding, NSB = non-specific binding.

MCF-7 cells were obtained from the Michigan Cancer Foundation. The growth of these cells has been shown to be inhibited by the addition of GnRH agonist [7]. MDA-MB-231 cells were from the American Tissue Culture Foundation. All cells were maintained at a temperature of 37°C in Dulbecco's minimal essential medium (DMEM) supplemented with heat-inactivated foetal calf serum (10%), sodium bicarbonate (16 mmol/l), penicillin (100 u/ml) and streptomycin (100 μg/ml) under a humidified atmosphere of 5% CO_2: 95% air. Cells were bulked up in Cellcult 150 cm^2 tissue culture flasks.

Rat pituitary glands were obtained from immature (28–35-day-old) female Sprague–Dawley rats killed by CO_2 asphyxiation. Glands were homogenised in ice-cold SET medium (0.5 ml/gland) using 12 strokes of a loose Dounce homogeniser, filtered through cheesecloth, snap-frozen in solid CO_2 in 2-ml aliquots, and stored at −20°C until required.

Placental tissue was obtained from women undergoing elective caesarian section at term. Tissues were homogenised and membrane fractions prepared as described previously [14].

Measurement of GnRH binding to tissue fractions

Triplicate aliquots (100 μl) of tumour fractions were incubated in a 0.5 or 1 ml system containing 40 mmol/l Tris–HCl, pH 7.4, 1% (w/v) BSA and 100 000 cpm of ^{125}I-labelled GnRH tracer. After incubation under the appropriate conditions (for rat pituitary and tumour homogenates, 2 or 4 h at 4°C, respectively; for human placenta, 1 h at 20°C), bound hormone was recovered by polyethyleneglycol precipitation [14, 16], and ^{125}I bound to the pellet counted in a Packard 'Crystal' gamma-counter at an efficiency of 75%. Non-specific binding was measured in duplicate in the presence of 1 μg (pituitary) or 10 μg (placenta and tumour tissues) unlabelled buserelin (normally 3–6% of total counts added for ^{125}I-labelled buserelin and 8–12% of total counts added for ^{125}I-labelled tryptorelin). The difference between binding in the presence and absence of unlabelled $GnRH_A$ was taken to represent specific binding (normally 10–45% total counts added), and was considered to be positive if Student's *t*-test (with the Bessel correction for small numbers) reached statistical significance. Controls without tissue, with and without unlabelled $GnRH_A$, were included to correct for displacement of tracer from assay tubes by cold analogue (1–4% of total counts added), and positive controls (rat pituitary; human placental membranes) were included in each binding assay.

Inactivation of GnRH tracers

Inactivation of radiolabelled GnRH tracers was measured as described previously [13, 14]. Briefly, quintuplicate aliquots of tissue fractions were incubated with 600 000 cpm of ^{125}I-labelled GnRH analogue or isoform tracer under the usual incubation conditions for each tissue. Tubes were chilled in ice and centrifuged at 30 000 *g* for 5 min. Supernatants were aspirated, and pellets and supernatants counted for ^{125}I in a gamma-counter (NE 1600) at an efficiency of 75%. The volume of each supernatant was adjusted with incubation buffer to give

90 000–100 000 cpm/ml, and triplicate aliquots (1 ml) were incubated with fresh human placental membranes for 1 h at 20°C, in the presence or absence of 10 μg unlabelled hormone to measure non-specific binding. After the second incubation, bound GnRH tracer was recovered by polyethyleneglycol precipitation, and pellets counted for ^{125}I. Three controls were always included: (a) tracer incubated with tissue at 0°C during the first incubation and incubation of tracer without tissue fractions at (b) 0°C or at (c) 20°C. All three supernatants were tested for binding in the second incubation, and values agreed to within ± 8%. Inactivation of GnRH tracer during the first incubation was reflected by a reduction in specific binding in the second incubation, and was expressed as percentage loss of binding relative to controls.

Protein was measured by the method of Lowry *et al.* [17].

RESULTS

Incubation of ^{125}I-labelled GnRH agonists with human breast tumour or benign breast tissue homogenates appeared to indicate low, but statistically significant, specific GnRH binding in a high proportion of breast tissue preparations (Table 1). However, unlabelled $GnRH_A$ also displaced similar amounts of tracer from control tubes in the absence of breast tissue homogenate (Table 1). Thus, after correction for displacement of ^{125}I-labelled GnRH agonist from control tubes, specific binding of GnRH to breast tumour tissues was no longer apparent in tissues which had previously appeared to show significant differences between binding in the presence and absence of unlabelled hormone (Table 2). Similar results were obtained with both buserelin and [D-Trp^6] GnRH ethylamide tracers (Table 2). Moreover, in contrast with GnRH agonist binding to human placenta and rat pituitary membranes, binding to human breast tissue homogenates did not increase consistently with increasing tissue concentration (data not shown).

Table 2. Binding of ^{125}I-labelled GnRH agonists to breast cancer, benign breast tissues and to rat pituitary and human placental membranes

Tissue	Number of samples with statistically significant binding of ^{125}I-labelled GnRH agonist tracer: Buserelin *n*	Buserelin Unadjusted	Buserelin Adjusted	[D-Trp^6] EtA *n*	[D-Trp^6] EtA Unadjusted	[D-Trp^6] EtA Adjusted
Human placenta	14	14	14	10	10	10
Rat pituitary	20	20	20	10	10	10
Benign breast	16	13	0	12	10	0
Breast cancer	43	32	0	32	15	0

Specific binding of ^{125}I-labelled GnRH agonist tracers in human placenta and rat pituitary membranes and human benign/cancerous breast tissues, in the presence or absence of 1 μg/tube (pituitary only) or 10 μg/tube of unlabelled buserelin, as described in Materials and Methods. Differences between binding in the presence and absence of cold hormone were calculated for each sample, and the statistical significance of the difference estimated by Student's *t*-test (unadjusted). Specific binding was recalculated for each sample following subtraction of the displacement of agonist tracer from control tubes with no tissue added (Adjusted). Positive control tissues (rat pituitary and human placenta) consistently showed specific agonist binding, but correction of agonist binding to breast tissues failed in every case to exceed significantly the displacement of tracer from tubes in the absence of homogenate.

Table 3. Inactivation of ^{125}I-labelled GnRH and GnRH agonist ($GnRH_A$) tracers during binding incubation

Tissue	Tracer bound (% total counts added) GnRH	Tracer bound (% total counts added) $GnRH_A$	Tracer inactivation (% control) GnRH	Tracer inactivation (% control) $GnRH_A$
Human placenta	6±1 (8)	18±2 (12)	72±6 (8)	31±4 (8)
Rat pituitary	4±0.6 (6)	14±2 (6)	57±10 (6)	17±3 (6)
Breast cancer	0 (5)	0 (5)	n.d.	9±2 (5)
MCF-7 cells	0 (3)	0 (3)	97 (2)	95 (2)
MDA-MB-231 cells	0 (2)	0 (2)	100 (2)	100 (2)

Triplicate aliquots (100 μl) of homogenates of human placenta, rat pituitary, breast cancer and breast cancer cells were incubated with 600 000 cpm of ^{125}I-labelled buserelin tracer and specific binding and tracer inactivation measured, as described in the Materials and Methods section. Specific binding was expressed as a percentage of total counts added, and inactivation of tracer as a percentage of control binding in the second incubation. Values are means ± SEM for *n* observations. n.d. = Not determined.

We determined whether the lack of consistent binding of GnRH tracers by breast tissue was due to inactivation of GnRH agonist tracer during the binding incubation. At the end of the incubation period, unbound tracer from systems containing breast material was recovered and re-incubated with fresh placental membranes. The results are shown in Table 3, and illustrate that 86–98% of the unbound ^{125}I-labelled GnRH agonist was still capable of rebinding to fresh placental membranes. MCF-7 and MDA-MB-231 cell lines did not bind GnRH agonist. However, exposure of GnRH and GnRH agonist tracers to homogenates of these cells almost completely abolished binding to placental membranes (Table 3). Inactivation of GnRH and GnRH agonist tracers by human placental and rat pituitary membranes were consistent with previously published data [13, 14].

We have shown recently that certain isoforms of GnRH (salmon GnRH and chicken GnRH II) can bind to human placental GnRH receptors, but not to rat or sheep pituitary receptors [14]. However, no isoform of GnRH bound significantly to human breast tumour tissue (data not shown).

DISCUSSION

Extrapituitary receptors for GnRH and its analogues have been demonstrated in a number of human tissues, including the placenta [14, 18–20], corpus luteum [16, 21] and ovary [22, 23]. These binding sites were of "low affinity" relative to pituitary receptors and did not discriminate between GnRH and GnRH agonist analogues. Similar "low affinity" GnRH binding sites have been demonstrated recently in human cancers of the prostate [24], endometrium [25], breast [11, 26] and ovary [27, 28].

Previous studies have described specific binding of radiolabelled GnRH analogues to human breast cancer cell lines and biopsy tissues [6–11, 26], and have demonstrated inhibitory effects of GnRH analogues on cell growth [6, 7, 10, 26]. These studies are difficult to compare, since there are many differences in the protocols and methodology used: namely different radiolabelled GnRH analogue tracers, tracer concentrations, incubation conditions, separation techniques to recover bound hormone, and concentrations of cold hormone to measure "non-specific" binding (and hence, calculation of "specific" binding).

Whilst some of these studies used techniques which clearly showed the presence of GnRH binding sites in breast tumours [26], other studies measured only low levels of binding [6, 9], although the majority of biopsy specimens were positive [11]. Other studies used such high concentrations of unlabelled hormone that non-specific displacement of tracer almost certainly occurred [6, 7], though corrections were not made. We also found a high proportion of apparent GnRH binding site-positive breast tissues in this study. However, the levels of binding observed were low (none in excess of 3000 cpm bound per mg protein). Furthermore, the concentration of unlabelled GnRH agonist which was required to saturate binding sites with a K_a of 10^7 mol/l [12] resulted in displacement of tracer from control tubes of a similar magnitude to the difference in binding to breast cancer tissues in the presence and absence of cold agonist. Thus, when differences between binding of GnRH agonists to breast tissues in the presence and absence of cold agonist were corrected for displacement from control tubes, no specific binding remained, though high levels of specific binding were demonstrable in all rat pituitary and human placental membrane preparations (Table 2).

We have shown recently that tissues which failed to bind GnRH agonist analogues specifically inactivated almost all the GnRH agonist tracer during the binding incubation [13, 14]. However, tracer inactivation did not contribute to the failure to demonstrate GnRH agonist binding to human breast cancer homogenates, as rebinding of unbound tracer to fresh placental membranes following exposure to breast cancer tissue was high (86–98%). In contrast, incubation of tracers with MCF-7 and MDA-MB-231 cell homogenates resulted in almost complete inactivation (Table 3), suggesting that (as for other tissues) high rates of tracer degradation may compromise the demonstration of GnRH receptors in these cells.

Human placental (and luteal) GnRH receptors differ in their specificity from pituitary GnRH receptors in that: (i) they do not discriminate between GnRH and superactive GnRH agonist analogues [14, 18–20]; (ii) they do not bind GnRH antagonists [13, 16, 29] and (iii) they bind salmon GnRH and chicken GnRH II, but not chicken GnRH I or lamprey GnRH [13, 14]. If human breast cancer cells have a similar GnRH receptor, they may also bind these GnRH isoforms. However, after correction for displaceable binding, no specific binding of these isoforms to human breast cancer tissue could be detected, despite binding of salmon GnRH and chicken GnRH II to human placental controls (data not shown). In conclusion, we could find no clear evidence for specific GnRH binding sites in human breast cancer or benign breast tissues.

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Oestrogen Receptor Message in Premalignant and Normal Cervical Cells: A Methodological Study

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Normal and abnormal biopsies of the uterine cervix, to a total of 124 samples, have been analysed for the detection of oestrogen receptor (ER) mRNA. The tough fibrous nature of the cervix proved very resistant to disaggregation in the first instance and subsequently, it was difficult to extract good high molecular weight message. This necessitated a systematic study of methodological technique, including two methods of tissue disaggregation and five techniques of extraction of ER mRNA, which in total involved the use of 124 cervical biopsies. The most successful method involved chopping the tissue, then digesting the cells with proteinase K and extracting the nucleic acids in salt and sodium dodecyl sulphate. Using the perfected technique, 16 cervical biopsies obtained at serial intervals from four women did not show any differences in ER mRNA in cervical biopsies either in the presence of oral contraception or histological abnormality. The successful method described will prove valuable for the detection of ER message in human tumours and other tissues of similar nature.
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INTRODUCTION

THE ASSOCIATION between human papillomavirus (HPV) and cervical cancer and precancer is well documented. Hybridisation studies using both Southern blot analysis [1] and polymerase chain reaction (PCR) [2] have indicated that there is a high rate of HPV16 detection in normal cervical tissues which suggests that cofactors other than HPV must influence cervical carcinogenesis. One such factor implicated in this context is smoking [3] which, as a putative promotor, could act on cervical cells in which abnormal cell changes had been initiated by viral transforming genes with the co-operation of activated oncogenes [4].

A hypothesis implicating oestrogen could be based on the following clinical observations. Cervical neoplasia generally arises in the so-called transformation zone (TZ) of the cervix which appears to be oestrogen-responsive. The TZ appears following the menarche, enlarges during the early reproductive era and pregnancy and then regresses postmenopausally. A source of exogenous oestrogen which could influence molecular events in an oestrogen responsive tissue is the combined oral contraceptive (OC), taken by millions of women of child-bearing age. In 1983, an epidemiological study appeared suggesting that long-term OC use conferred an increased risk of developing cervical cancer [5].

Previous reports from our laboratory showed that the level of transcription of the oestrogen receptor (ER) in oestrogen responsive cells could be increased, not only by exogenous oestrogen, but also by infection with herpes simplex virus (HSV). Moreover, the increase in the ER message in *in vitro* studies was shown to be induced by the viron trans-inducing factor (TIF) or Vmw65 [6], a component of the HSV virion. Exogenous oestrogen has been shown to be an obligatory requirement for the oncogenicity of MCF-7 cells in the nude mouse. In the absence of such oestrogenic stimulation, oncogenicity is only effected after transfection of MCF-7 cells with the oncogene V-*ras*^{H} [7].

The HSV TIF has also been shown to stimulate the early E7 ORF of HPV18 [8] whose expression is correlated with

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