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Report

Characterization of binding sites for a GnRH-agonist (buserelin) in human breast cancer biopsies and their distribution in relation to tumor parameters

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Summary

Gonadotropin-releasing hormone analogs (GnRH-A) have been added to the armentarium in the therapy of hormone-dependent breast cancer in premenopausal women. The effect of chronic GnRH-A-treatment in premenopausal women is based on the suppression of the hypothalamus-pituitary-ovarian axis and the reduction of sex-steroid serum levels. In addition, a number of experimental and clinical data have been accumulated indicating a direct action of GnRH-A on breast cancer cells and tissue. In this study we analyzed 235 human breast cancer biopsies for specific GnRH-A-binding. We demonstrate high affinity GnRH-A binding sites in human breast cancer tissues. The evaluation of clinical data showed no correlation of the level of GnRH-A-binding with classical tumor parameters.

Introduction

The use of gonadotropin-releasing hormone analogs (GnRH-A) in the therapy of advanced breast cancer in premenopausal women has been established in the last years [1–3]. The success of this therapeutic regimen is due to the suppression of the ovarian function as a result of the desensitization of the pituitary gland [4, 5]. In addition, evidence has been accumulated that GnRH-A may also exert antiproliferative effects directly on breast cancer tissue and cell lines. Despite some contradictory studies [6, 7], the clinical benefits in some postmenopausal women with advanced breast cancer following treatment with GnRH-agonist [8, 9] indicate a potential mechanism of growth inhibitory action of GnRH-A that is independent of the suppression of the ovarian steroid synthesis and secretion.

The first step of GnRH-A action is the binding to a specific receptor. In different types of human cancer tissues, i.e. in pancreatic [10], endometrial [11], and ovarian carcinomas [12], as well as in breast cancer cell lines and tissues [13–17], GnRH-A binding sites have been demonstrated. The biological significance is further underlined by *in vitro* and *in vivo* studies demonstrating the growth inhibitory effects of GnRH-A [18–23]. The exact mechanisms by which GnRH-A mediate their antiproliferative effects are not yet clear. Investigations in hormone-dependent and -independent breast cancer cell lines support the hypothesis that the hydrolysis of phospholipids and the activation of protein kinase C [24] may be involved.

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Further studies are necessary to support the clinical efficacy GnRH-A. In this study high affinity GnRH-A-binding sites in human mammary cancer tissues were characterized and a large series of breast cancer biopsies was investigated for specific GnRH-A binding.

Material and methods

Peptides

The GnRH-agonist buserelin [pGlu-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-NH-Et] and thyrotropin-releasing hormone were a gift from Hoechst AG (Frankfurt/Main, Germany). Iodinated buserelin (specific activity of approximately 1400 μCi/μg) was kindly provided by Dr. J. Sandow (Hoechst AG, Frankfurt/Main); buserelin was labelled by the modified chloramine-T-method [25] according to Greenwood *et al.* [26]. Angiotensin II was obtained from Boehringer Mannheim (Mannheim, Germany).

Buffers

Homogenization and preparation of membranes were performed in 10 mM Tris/HCl buffer, pH 7.4 (buffer A). Buffer B used in the receptor assay was prepared by addition of 0.1% bovine serum albumin (Sigman, München, Germany) to buffer A.

Preparation of rat pituitary membranes

Pituitaries of female Sprague Dawley rats (Süddeutsche Tierfarm, Germany) served as positive controls for the GnRH-A binding assay. The pituitaries were frozen in liquid nitrogen immediately after removal and stored at −80° C until preparation of the membrane fraction. One pituitary was homogenized in 1 ml buffer A using a polytron homogenizer (five strokes for 7-sec duration at highest speed). One ml buffer A was added to the homogenate and was centrifuged at 800 xg for 10 min at 4° C. The supernatant was centrifuged at 10,500 xg for 45 min at 4° C. The resulting pellet was resuspended in 1 ml of buffer A and used for the binding assay. Six pituitaries were pooled for characterizing GnRH-A binding. All preparation procedures were carried out on ice.

Preparation of human breast cancer membranes

Breast cancer biopsies from pre- and postmenopausal women were divided into separate specimens for histological examination, steroid receptor measurement, and GnRH-A-receptor binding assay immediately after surgical removal. Specimens used for GnRH-A binding investigations were stored at −80° C until further use. The membrane preparation of the breast cancer samples was performed in the same way as the preparation of rat pituitary membranes. In a first experiment the samples were screened for specific GnRH-A-binding. For binding characterization experiments, tissue samples with detectable specific GnRH-A-binding were pooled.

GnRH-A-binding assay

Membrane preparations were incubated with the radioiodinated GnRH-A buserelin (60–100 pM) and various concentrations of unlabelled peptide in buffer B up to a final volume of 0.5 ml. Assay mixtures were incubated for 120 min at 4° C in triplicate. The reaction was terminated by adding 0.5 ml bovine gamma globulin (0.1%) and 1 ml polyethylene glycol (25%) (all from Serva, Heidelberg, Germany). The tubes were vortexed carefully. The bound radioactivity was precipitated by centrifugation at 1,600 xg for 15 min at 4° C. The supernatant was aspirated and the pellet counted in a gamma counter.

Protein determination

The protein content of the membrane preparations was measured by the method of Bradford [27] using the Bio Rad Protein Assay (Bio Rad, München, Germany). The protein concentration was adjusted

to 0.5 to 0.8 mg/ml. Bovine serum albumin was used for standardization.

Analysis of the binding data

Specific ^{125}I-buserelin-binding was determined in the presence of 10μM unlabelled buserelin. The characterization of the binding sites (affinity constants [K_A] and maximal binding capacities [B_{max}]) was calculated by the method of Scatchard [28] with the modifications by Chamness and McGuire [29] using a computerized mathematical regression analysis.

Specific GnRH-A-binding to breast cancer specimens was characterized by a specific binding content of ^{125}I-buserelin ≥300cpm [30], a GnRH-A binding content of ^{125}I-buserelin ≥3fmoles/mg membrane protein, and a statistically significant (t-test ≤0.05) difference between total and non-specific ^{125}I-buserelin-binding.

The distributions of the GnRH-A binding content into subgroups of tumor samples characterized by classical tumor parameters are represented in box plots. In the box plot, the box contains 50% of the data, and the line within the box shows the median. The distant ends of the upper and lower vertical lines connecting the values outside the box with the upper and lower hinges represent the 90th and 10th percentiles, respectively [31].

Statistical analysis

Where indicated, the Student's t-test, the Kendall's rank correlation test, or the Wilcoxon-Mann U-test were performed using a Statview (Abacus Concepts, USA) program.

Results

Time dependence of specific GnRH-A-binding

The membrane preparations of rat pituitaries and human breast cancer biopsies were incubated up to 360 min at 4° C with ^{125}I-buserelin (60pM), and unlabelled peptide (10μM) to determine non-specific binding. The maximal specific GnRH-A-binding in the pituitary and in breast cancer membranes was achieved after 90 min. A significant decrease in specific GnRH-A-binding content was detectable in the membranes of the pituitary after 180 min (not shown), whereas in the human breast cancer membranes the specific GnRH-A-binding was stable up to 360 min (Fig. 1). For further experiments, the incubation time of 120 min was chosen as being in the steady state of the binding reaction.

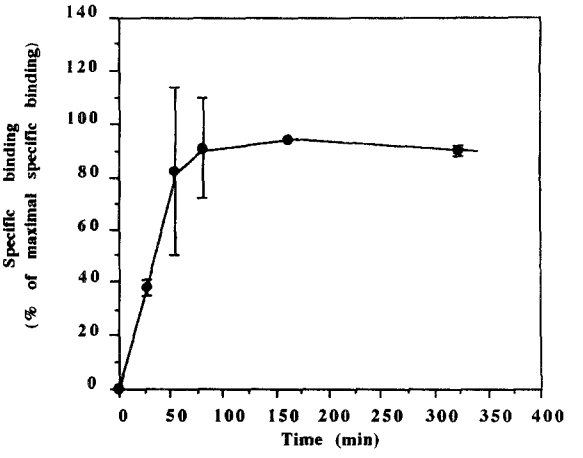

Fig. 1. Time dependence of specific ^{125}I-GnRH-A-binding to human breast cancer membranes. Preparations of human breast cancer membranes (60μg) were incubated with 70pM ^{125}I-buserelin and the reaction was terminated after different periods of time. Non-specific binding was determined in the presence of 10μM unlabelled buserelin. The data represents two independent experiments.

Saturation of specific GnRH-A-binding

Increasing concentrations of ^{125}I-buserelin from 1pM up to 0.5nM were incubated with membrane preparations of rat pituitaries or human breast cancer tissues. The non-specific binding was determined in the presence of 10μM unlabelled buserelin. The specific GnRH-A-binding was shown to be saturable (Fig. 2). In the representative experiment shown in Fig. 2, saturation of specific GnRH-A-binding in breast cancer tissue was achieved at a concentration of approximately 200pM iodinated

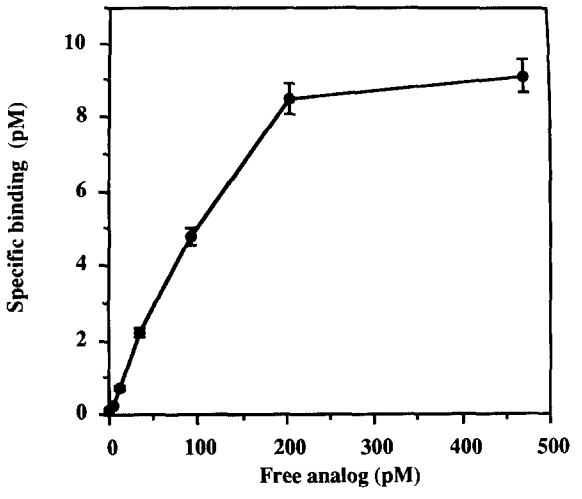

Fig. 2. Saturation of specific ^{125}I-buserelin-binding to human breast cancer membranes. Increasing concentrations of ^{125}I-buserelin (from 1pM to 0.5nM) were incubated with preparations of human breast cancer membranes for 120 min. The non-specific binding was determined in the presence of 10μM unlabelled buserelin.

buserelin (saturation of GnRH-A binding to rat pituitary membranes was obtained with 90pM iodinated buserelin).

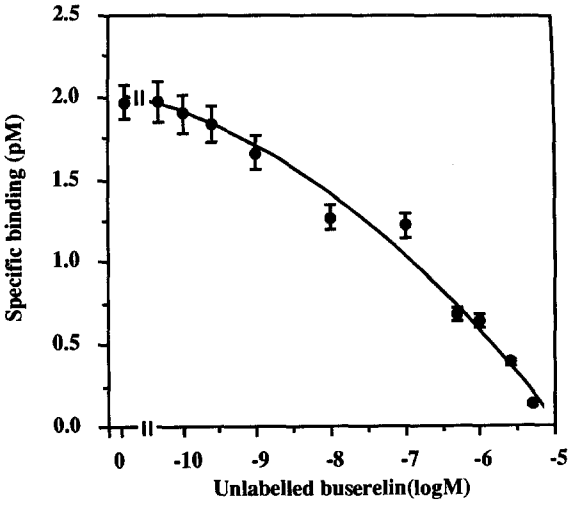

Fig. 3. Displacement of ^{125}I-buserelin-binding to human breast cancer membranes. Increasing concentrations of unlabelled buserelin (from 50pM to 5.0μM) were incubated with preparations of human breast cancer membranes for 120 min. The concentration of labelled buserelin was 75pM.

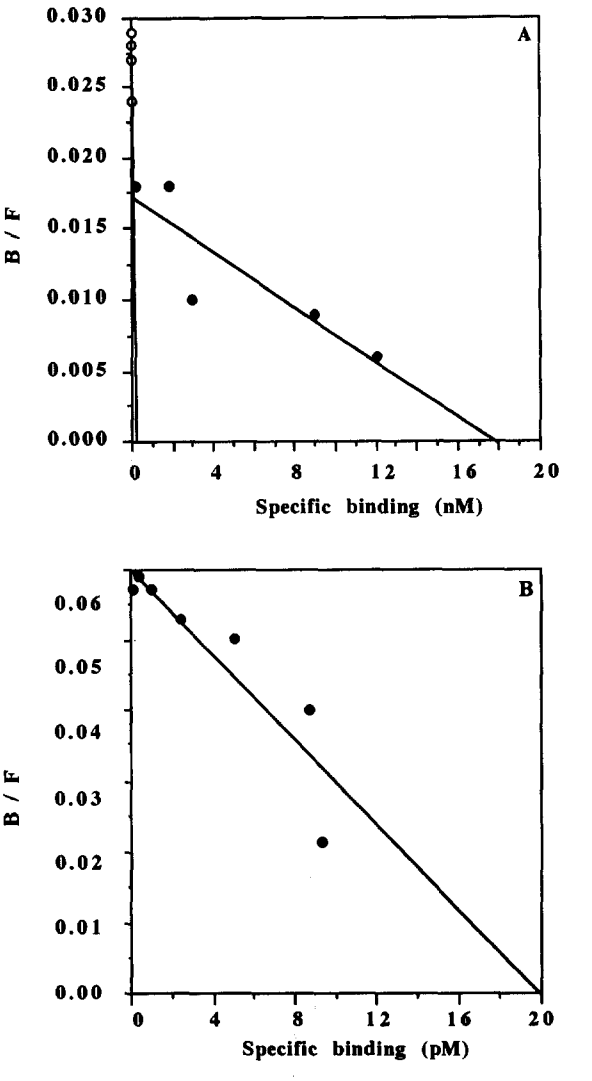

Fig. 4. Scatchard plot analysis of buserelin binding to human breast cancer membranes. A: Data from the displacement experiment shown in Fig. 3. B: Data from the saturation experiment shown in Fig. 2 representing the high affinity binding site for the GnRH-agonist. B = bound; F = free.

Displacement of specific GnRH-A-binding

In homologous displacement experiments, increasing concentrations of unlabelled buserelin (from 50pM to 5μM were incubated with membrane preparations of rat pituitaries and human breast cancer tissues (Fig. 3) in the presence of a constant concentration of radiolabelled buserelin (75pM). The bound radioactivity in the presence of 10μM

Table 1. Affinity constants (K_A) and maximal binding capacities (B_{max}) of the binding of the gonadotropin-releasing hormone-agonist buserelin to human breast cancer and rat pituitary membranes

Tissue (number of experiments)	High affinity binding site		Low affinity binding site	
	K_A	B_{max}	K_A	B_{max}
Rat pituitary (n = 10)	$1.1 \times 10^9 M^{-1}$	334pM	$5.1 \times 10^6 M^{-1}$	10.7nM
Human breast cancer (n = 18)	$2.5 \times 10^9 M^{-1}$	100pM	$71 \times 10^6 M^{-1}$	17.5nM

unlabelled buserelin was assessed as non-specific. The inhibition of 50% of ^{125}I-buserelin binding to the membranes of rat pituitaries and human breast cancer tissues was obtained by 10nM and 60nM unlabelled buserelin (Fig. 3), respectively.

Scatchard analysis of binding data

The data of the displacement experiment shown in Fig. 3 have been transformed by the method of Scatchard [28] modified by Chamness and McGuire [29] in a computerized mathematical regression analysis (Fig. 4A). The linear transformation revealed two classes of GnRH-A-binding sites (Table 1). The values of the affinity constants of the GnRH-A high affinity binding sites in pituitaries and human breast cancers were close together. For the high affinity GnRH-A binding site, the maximal GnRH-A-binding capacity of the membrane preparations of the pituitary (0.3nM) was higher than that of human breast cancer (0.1nM).

Specificity of GnRH-A-binding

^{125}I-buserelin (80pM) bound to membranes prepared from pooled breast cancer tissues was not displaced from the binding sites by high concentrations of unrelated peptides. Angiotensin II and thyrotropin releasing hormone in concentrations up to 10μM did not have any effect on ^{125}I-buserelin binding (Fig. 5). Additionally, Fig. 5 shows an example of a breast cancer sample without detectable specific GnRH-binding sites.

Evaluation of a series of 235 breast cancer samples

In this series, biopsies of 235 female patients with primary breast cancer were investigated for specific GnRH-A-binding. The characteristics of the patients are shown in Table 2, including the data for the subgroups of pre- and postmenopausal patients. In 121 (51.5%) specimens out of 235 samples, specific GnRH-A-binding sites were detectable.

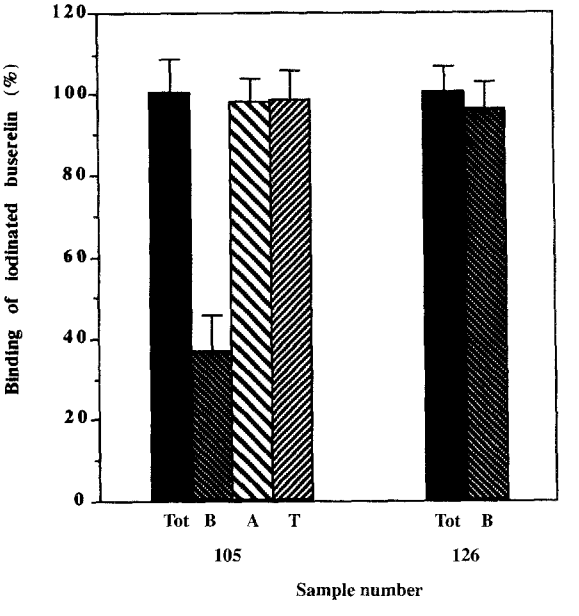

Fig. 5. Peptides not related to the gonadotropin-releasing hormone agonist buserelin did not displace iodinated buserelin from binding sites (e.g. breast cancer sample 105). 10μM unlabelled buserelin (B), angiotensin II (A), or thyrotropin-releasing hormone (T) were incubated with human breast cancer membranes for 120 min in the presence of labelled buserelin (65pM); total binding (Tot). Breast cancer sample 126 represents a tissue sample without detectable GnRH-A binding sites.

Table 2. Characteristics of 235 breast cancer patients and tissues. GnRH-A = Gonadotropin-releasing hormone-analog. SD = Standard deviation; SEM = Standard error of the mean

Patients	Age (years ± SD)	Number	GnRH-A-binding (fmol/mg ± SEM)	Samples with a GnRH-A-binding content ≥3 fmol/mg n (%)
All	55.8 ± 13.4	235	6.4 ± 0.5	121 (51.5)
Premenopausal	43.2 ± 6.0	95	5.6 ± 0.8	42 (44.2)
Postmenopausal	64.3 ± 9.8	140	6.9 ± 0.7	79 (56.4)
Estrogen receptor positive		126	6.1 ± 0.6	69 (54.8)
Estrogen receptor negative		106	6.4 ± 0.9	49 (46.2)
Progesterone receptor positive		120	6.2 ± 0.7	61 (50.8)
Progesterone receptor negative		111	6.3 ± 0.8	57 (51.4)
Ductal invasive		175	6.8 ± 0.7	93 (53.1)
Lobular		39	5.3 ± 1.0	20 (51.3)

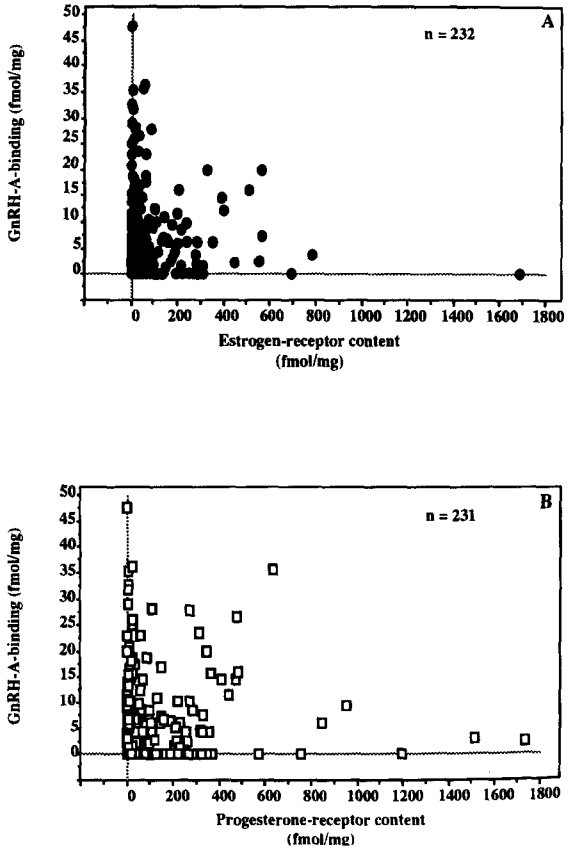

Fig. 6. The gonadotropin-releasing hormone-analog (GnRH-A)-binding content of the breast cancer samples is drawn against the estrogen (A) and progesterone (B) receptor content.

The relation between the steroid receptor content and the GnRH-A binding values was analyzed (Fig. 6). No correlation was found between the estrogen or progesterone receptor content and the GnRH-A-binding level (Table 3). The GnRH-A binding concent in steroid receptor-positive or -negative samples (Table 2) was not significantly different (U-test).

Histologically, 175 cancer biopsies were characterized as ductal-invasive, while 39 were classified as lobular carcinomas. The remaining breast tumors represented other types of malignant breast tumors. The distribution of the GnRH-A-binding content in lobular and ductal breast carcinomas is

Table 3. Correlation coefficients (τ, Kendall's rank correlation test) between the binding capacities of estrogen, progesterone, and gonadotropin-releasing hormone analog (GnRH-A) in human breast cancer membranes. n = number of samples. NS = no statistically significant correlation

Receptors for	Estrogen	Progesterone	GnRH-A
Estrogen	–	$\tau = 0.448$ $p < 0.05$ n = 231	$\tau = 0.026$ NS n = 232
Progesterone	$\tau = 0.448$ $p < 0.05$ n = 231	–	$\tau = -0.006$ NS n = 231
GnRH-A	$\tau = 0.026$ NS n = 232	$\tau = -0.006$ NS n = 231	–

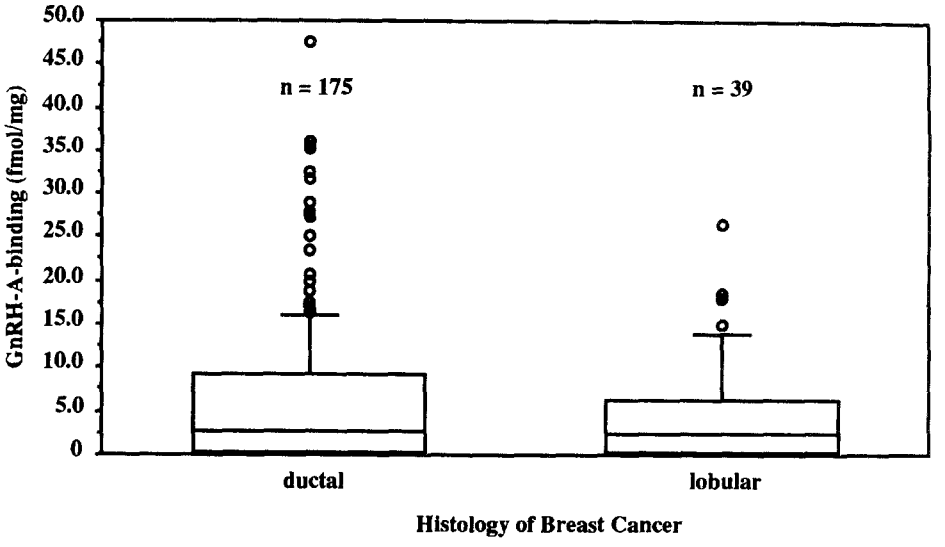

Fig. 7. Distribution of gonadotropin-releasing hormone analog (GnRH-A)-binding content in ductal and lobular human breast cancer biopsies.

shown in Fig. 7. No significant difference in respect to the GnRH-A-binding (Table 2) was revealed for the two groups (U-test).

In respect to the TNM-tumor classification, no significant difference in GnRH-A-binding could be evaluated in the different groups. In 165 breast tumor biopsies with known nodal (N) status, the mean GnRH-A-binding content was not significantly different (U-test) for the N0, N1, N2, and N3 groups (Fig. 8).

The S-phase index as a marker for mitotic activity was available for 129 breast cancer specimens. The

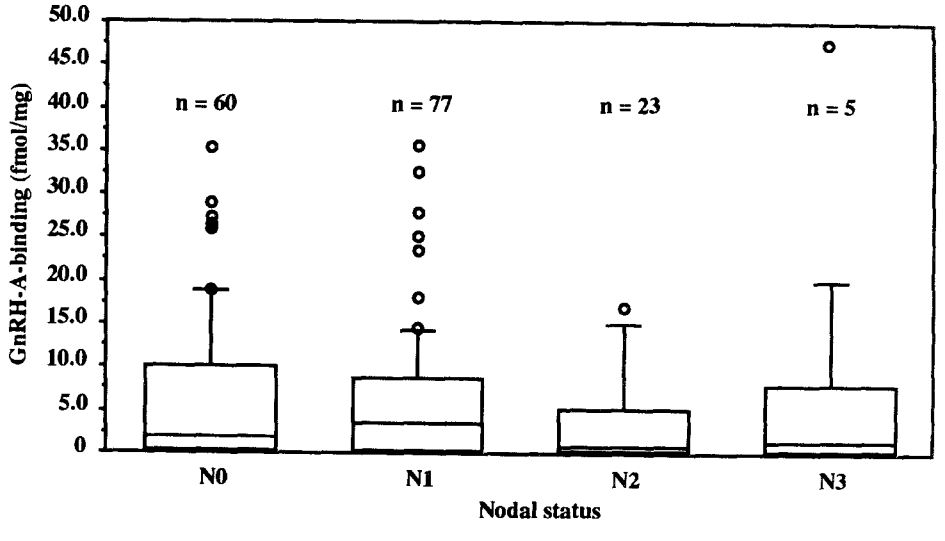

Fig. 8. Distribution of gonadotropin-releasing hormone analog (GnRH-A)-binding content in human breast cancer biopsies. The groups were characterized by nodal status.

samples were grouped into an S-phase index <5% group (n=74) and an S-phase index ≥5% group (n=55). The GnRH-A-binding in the two groups did not differ significantly (U-test).

Discussion

Analogs of the decapeptide GnRH are widely used in the treatment of hormone-dependent prostate, pancreatic, and breast cancer [2, 3, 32–34]. Due to the downregulation of pituitary and the following suppression of gonadal function, hormone-dependent cancers are deprived of one of their main mitogenic stimuli. The low level of side effects of agonistic GnRH-A has allowed these peptides to become an important tool in hormone-dependent cancer treatment [35, 36].

In addition to the inhibition of the pituitary-gonadal function by GnRH-A, a number of *in vitro* [13, 18, 20], *in vivo* [22, 23, 37], and clinical [8, 9] data give evidence of an antiproliferative activity of GnRH-A that cannot be explained by the suppression of gonadal function. GnRH-A may exert growth inhibitory effects directly on the cancer cells.

In many extrapituitary human tissues, GnRH-A-binding sites have been described [38–41]. The characterization of high affinity binding sites of GnRH-A in human breast, pancreatic, and endometrial cancers [10, 11, 14] has further supported the earlier detection of specific GnRH-A-binding sites in human cancer tissues [12, 13, 15]. In our own study, we characterized two classes of GnRH-A-binding sites in human breast cancer tissues. We compared our results with the GnRH-A-binding characteristics to rat pituitary membranes. The maximal binding capacity of the high affinity GnRH-A-receptor in the pituitary was higher than that of the cancer samples. Our calculated binding data for high affinity GnRH-A-binding to membranes of human breast cancer tissues are in close agreement with the values found by Fekete *et al.* [14].

The regression following GnRH-agonist or -antagonist treatment of hormone-dependent cancers in animals may be mediated by these high affinity GnRH-A-binding sites [22, 23]. Effects on the course of breast cancer in postmenopausal women using GnRH-agonists [8, 9] may also be a result of the specific binding of GnRH-A to specific binding proteins in the membrane of the cancer cells. The presence of GnRH-A-binding sites, the absence of steroid receptors, and a benefit of the cancer patients by GnRH-A-treatment may support the hypothesis of a direct antiproliferative potency of GnRH-A.

The demonstration of the activation of intracellular signal transduction systems (e.g. increased hydrolysis of phospholipids; activation of protein kinase C) in human breast cancer cell lines by GnRH-agonists underlines that the GnRH-A-binding sites may represent functional receptors [24, 42]. In addition, the increase of the messenger ribonucleic acid of p21, a protein mainly regulated by estrogen, after treatment with a GnRH-agonist supports the view of a direct regulatory effect of GnRH-A on cancer cells and tissues [43]. However, the exact mechanism by which GnRH-analogs influence cell growth is not yet understood. A further insight on the regulatory significance of the GnRH-A binding sites comes from the demonstration of GnRH-like petides in human breast cancer biopsies and cell lines [44, 45], which further supports a model of a direct effect of GnRH-A on breast cancer, although a growth regulatory function of these GnRH-like petides has not yet been investigated.

The clinical relevance of the measurement of GnRH-A-binding sites in breast cancer tissues might be further indicated by the use of cytotoxic drugs coupled to GnRH-A accumulating in cancer tissues expressing GnRH-A-binding sites [36, 46]. Due to the detection of GnRH-A binding sites in cancer biopsies, patients might be selected for this kind of drug targeting therapy in the future. A follow-up of the clinical course of cancer patients whose tumor biopsies were investigated for GnRH-A binding might elucidate whether the expression of GnRH-A binding sites can serve as a parameter for therapeutic decision-making and/or as a prognostic factor.

The use of GnRH-A for breast cancer treatment has been established in premenopausal patients with advanced breast cancer. In addition, strong

evidence has been accumulated regarding the direct antiproliferative effects of GnRH-A on cancer tissues via specific binding sites. A step towards clinical significance has been undertaken by the strategy of using cytotoxic drugs coupled to GnRH-A to reduce the cytotoxic side effects. The measurement of GnRH-A-binding by radio-receptor-assay could serve as an indicator for a successful use of the different GnRH-A-treatment schedules. Further studies need to evaluate the relevance of GnRH-A binding as a prognostic factor and its use as a parameter for the choice of treatment.

Acknowledgements

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