Characterization of Bombesin/Gastrin-releasing Peptide Receptors in Human Breast Cancer and Their Relationship to Steroid Receptor Expression

Gabor Halmos, James L. Wittliff, and Andrew V. Schally

ABSTRACT

Bombesin (BN) and its mammalian counterpart, gastrin-releasing peptide (GRP), are hormonally active peptides which appear to function as autocrine or paracrine growth factors in a variety of cells. As part of a long-term investigation of the relationship of peptide and steroid hormone receptors to breast cancer progression and treatment, we examined the binding of [125I-Tyr4]BN to membranes isolated from 100 human breast carcinomas. Thirty-three of these tumors expressed BN/GRP receptor levels of >10 fmol/mg membrane protein. Two classes of [Tyr4]BN-binding sites were detected using Scatchard analyses of radioligand association data from hormone displacement curves. The high-affinity binding sites exhibited a mean dissociation constant (K_d) of 2.1 nM and a mean specific binding capacity (B_max) of 237 fmol/mg membrane protein. The low affinity binding sites had a mean dissociation constant (K_d) of 0.3 μM and a mean binding capacity (B_max) of 5.9 pmol/mg membrane protein. BN/GRP receptor expression in a breast carcinoma was unrelated to patient age. When the levels of BN/GRP receptors were compared to the content of the sex steroid receptors, a highly significant positive correlation (P < 0.005) was observed between the binding capacities of high-affinity [Tyr4]BN-binding sites and estrogen receptor levels and between the concentrations of low affinity [Tyr4]BN-binding sites and progesterin receptor levels (P < 0.05). This represents the first report of these labile, regulatory proteins in biopsies of human breast carcinomas. Expression of specific receptor proteins for BN/GRP, potent mitogens, in a large number of human breast cancers suggests that they may be involved in tumor cell progression. The approach based on determination of BN/GRP receptors might be useful to guide a hormonal therapy with BN/GRP antagonists in some women with breast cancer.

INTRODUCTION

The differentiation and development of the human breast including its function during pregnancy and lactation are regulated by a variety of hormones including 17β-estradiol and progesterone, cortisol, the thyroid hormones, and certain peptide hormones, such as prolactin, growth hormone, and oxytocin (1). There is increasing evidence that growth factors, particularly epidermal growth factor, are involved in development and function of normal and neoplastic breast (2). Estrogens and EGF may influence the mitotic state of mammary epithelial cells and appear to be involved in ductal development, while progesterone promotes differentiation of these cells resulting in lobular development. Prolactin acts synergistically with estrogen and progesterin to bring about ductal and lobuloalveolar cell development. Prolactin is also the principal hormone that stimulates lactogenesis. The exact roles of the other hormones are less well understood, and most of our knowledge is derived from studies in experimental animals (5).

Breast carcinoma, a disease of the mammary epithelium, is the second leading cause of cancer-related deaths among American women (6). In 1993, more than 180,000 new cases of breast carcinoma were diagnosed in women in the United States alone. Approximately 48,000 women in the United States will die from this disease in 1994 (6). More than 1 million women in America are currently living with stage I, II, or III breast cancer, which indicates the magnitude of this health problem.

Approximately 30% of women who present with node-negative primary breast cancer are at high risk for early recurrence of metastatic disease and decreased overall survival (7). Although most breast cancer patients with node-positive disease are at high risk for decreased disease-free survival and overall survival, 30% appear to be at low risk, considering their clinical course. One of the objectives in the application of molecular endocrinology to the problem of breast cancer is to distinguish these high risk patients from those with low risk using clinical laboratory tests (7, 8).

The presence of estrogen and progesterin receptors in a breast tumor biopsy serves as a predictive indicator of therapeutic response to endocrine treatments, such as tamoxifen and medroxyprogesterone acetate (9, 10). These receptor proteins are also prognostic indices of a patient’s clinical course (7, 8). Recently, overexpression of EGF receptors in breast cancer (2, 4) has been correlated with decreased disease-free interval and overall survival (3, 11). Thus, these receptors and other cellular expression products appear to be useful as prognostic factors in clinical management of breast cancer.

As part of a long-term investigation to define the molecular mechanisms of endocrine control of breast cancer, we have systematically evaluated human tumor biopsies for hormone receptors. In addition to extensive evaluation of sex steroid receptors (8, 12), we detected prolactin receptors in human breast cancer (13) although no clinical utility of this finding could be demonstrated thus far. We have likewise characterized EGF receptors in human breast (4), endometrial (14), and ovarian carcinomas. Specific, high-affinity binding sites for luteinizing hormone-releasing hormone and somatostatin were also identified in human mammary tumor cell lines and in biopsy specimens of breast cancer by our group (4, 14) and by others (15). Our long-term goal is to exploit the presence of hormone binding mechanisms for new therapeutic approaches in breast and other endocrine-responsive cancers.

Recent evidence suggests that tetradecapeptide bombesin, originally isolated from amphibia, and its mammalian counterpart gastrin-releasing peptide can function as growth factors, and through an autocrine or paracrine mechanism it may modulate the growth of some benign and neoplastic tissues (18, 19). BN-like peptides function as potent mitogens in vitro and have been implicated as autocrine growth factors in the pathogenesis and progression of some human small cell lung carcinomas (18, 20, 21). Various studies also demonstrated that bombesin/GRP may be involved in the function and

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3 The abbreviations used are: EGF, epidermal growth factor; GRP, gastrin-releasing peptide; BN, bombesin; ER, estrogen receptor; PR, progesterin receptor; BNR, [Tyr4]-bombesin receptor.
growth of human breast cancer (22—27). BN-like immunoreactivity has been reported in rat mammary tumors (28), in a small proportion of human breast cancer and breast carcinoid specimens (25), in human and animal milk (29), and in normal bovine mammary epithelium (19). It has also been shown that the BN/GRP antagonist RC-3095 inhibits growth of estrogen-dependent and -independent MXT mammary cancer in mice (30) and MCF-7 MIII human breast cancer lines in nude mice (31). In addition, specific receptors for bombesin/GRP have been demonstrated in human breast tumor cell lines, MDA-MB-231, MCF-7 MIII, and T47D, but were not detected in MCF-7 and BT-20 cell lines or in normal breast epithelial cells (23, 31, 32).

Three receptor subtypes associated with the bombesin-like peptides have been described and cloned. The cloned receptors have been distinguished by their relative affinity for binding a series of specific ligands. They are classified as: the GRP-prefering subtype (GRP receptor), found in the intestine from the esophagus to the rectum (19); the neuromedin B-prefering subtype, present in the esophageal muscularis mucosa (33); and the bombesin receptor subtype 3, present in the testis and lung cancer cell lines (21), the natural ligand of which is not yet known. GRP receptor subtypes bind GRP and bombesin with high affinity and neuromedin B with lower affinity. In contrast, the neuromedin B receptor subtype has a higher affinity for neuromedin B than for GRP or bombesin (34). All three subtypes belong to the serpentine receptor superfamily and are coupled through G proteins to effectors including phospholipase C.

Collectively these observations strongly suggest the need for evaluation and characterization of bombesin/GRP receptors in human breast cancer biopsies to enhance our understanding of the mechanisms involved in these responses. In this study, we report for the first time the presence of specific high-affinity binding sites for [Tyr]BN in human breast cancer specimens. Biopsy specimens from 100 human breast cancers were analyzed simultaneously for [Tyr]BN binding sites on membrane preparations and estrogen and progesterone receptors in cytosol in order to correlate the levels of expression of these receptors.

**MATERIALS AND METHODS**

**Chemicals.** [Tyr]BN was purchased from Bachem (Torrance, CA) while [125I-Tyr]bombesin was prepared in our laboratory using radioisotope 125I-labeled sodium from Amersham (Arlington Heights, IL). 17β-[H]estradiol and [H]R5020 were purchased from NEN Products/DuPont (Wilmington, DE). Other peptides and chemicals were obtained from Sigma (St. Louis, MO), Pierce (Rockford, IL), Baxter (Muskegon, MI), Debiopharm SA (Lausanne, Switzerland), or American Peptide Company, Inc. (Sunnyvale, CA).

**Preparation of Breast Cancer Specimens.** Biopsies of human breast cancer were submitted for clinical determinations of sex hormone receptors. All analyses were first conducted to meet the primary clinical requirement for patient management; only residual tissue was used for this study. After surgical removal, a histopathological examination of each specimen was performed immediately, and a portion of the biopsy was frozen on dry ice and stored at −80°C until the analyses of steroid and peptide hormone receptors. Patient age ranged from 29 to 94 years.

**Determination of Estrogen and Progestin Receptors.** Estrogen and progesterone receptors were determined by radioisladig binding using a titration assay (35). Briefly, cytosol was prepared as described using 10 mM Tris-Cl buffer (pH 7.4), containing 1.5 mM EDTA, 10 mM monothioglycolic acid, 10% glycerol, and 10 mM sodium molybdate. Protein concentrations of the cytosol were adjusted to 2 mg/ml and incubated with either 17β-[H]estradiol or [H]R5020 at 6 concentrations ranging from 0.2 to 4 nM at 4°C for 16—18 h. Nonspecific binding was assessed in the presence of a 200-fold excess of either unlabeled diethylstilbestrol or R5020 for estrogen and progesterone receptors, respectively. Unbound hormone was separated from bound labeled steroid by incubation with 0.3 ml of a dextran-coated charcoal suspension for 15 min followed by centrifugation. All assays were performed in duplicate, and specific binding capacities and apparent dissociation constants (K_d) were calculated according to the procedure of Scatchard (36) using Lundoon One-Site software (Lundon Software, Inc., Cleveland, OH). Using clinical criteria established by the National Surgical Adjuvant Breast and Bowel Project (37), tumors containing at least 10 fmol/mg cytosol protein were considered positive for the sex hormone receptors.

**Membrane Preparation.** Portions of intact human breast cancer specimens (300—400 mg each) were shipped on dry ice from the Hormone Receptor Laboratory, University of Louisville (where estrogen and progesterone receptors were determined), to the Endocrine, Polypeptide and Cancer Institute in New Orleans, where the membrane receptor assays were performed. Preparation of membranes for receptor studies was carried out as described previously (4, 38) with some modifications. The samples were thawed and fat debris was removed using cotton swabs. Immediately after this, tumor specimens were homogenized (1:5, v/v) in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA-5 mM MgCl2-30 µg/ml bacitracin on ice using an Ultra-Turrax (IKA-Werk-Tekmar, Cincinnati, OH) homogenizer at maximal speed (stressed 3 times for 5 s at 15-s intervals). The homogenate was centrifuged at 500 × g for 10 min at 4°C, and the lipid layer was removed carefully. The supernatant containing the crude membrane fraction was centrifuged again at 70,000 × g for 50 min at 4°C in a Beckman LS-80 M ultracentrifuge. The pellet was washed once by first resuspending it in an ice-cold homogenization buffer and then sedimenting it as before. The final pellet was resuspended in homogenization buffer and stored at −80°C until used for the bombesin receptor binding studies. Preliminary results are available regarding the stability of these receptors to freezing since they have been described only recently (35). During our study, bombesin binding was performed immediately after membrane preparation and again 3—4 weeks later as a control in biopsies with sufficient tissue. No significant changes in either K_d or receptor levels were observed. The storage of human breast tumor biopsies at −86°C is well accepted for retarding the degradation of steroid and peptide hormone receptors and certain enzymes (4, 8). Protein concentration was determined by the method of Bradford (39) from free ligand by centrifugation at 4500 × g for 10 mm at 4°C. The pellet was resuspended in fresh binding buffer and ultracentrifuged, the final pellet was resuspended in the original binding buffer and homogenized by a Vortex. After centrifugation, the supernatant was removed, the pellet was resuspended in an equal volume of fresh binding buffer and ultracentrifuged. The supernatant was removed and the pellet was resuspended in an equal volume of fresh binding buffer. The binding activity was measured by adding 100—200 µCi of [125I-Tyr]BN to 100—200 µCi of purified [125I-Tyr]BN. Since the mono- and diiodinated [125I-Tyr]BNs were isolated in pure form, separated from each other and from the noniodinated peptide and oxidation products, a theoretical specific activity of 1800—2000 Ci/mmol was calculated. The adsorption of mono- [125I-Tyr]BN to different types of surfaces such as borosilicate glass, polystyrene, polypropylene, Aquasil-coated glass, Sigma coated glass, and siliconized polypropylene was evaluated. Since essentially no surface adsorption occurred using borosilicate glass tubes, these tubes offered the most favorable conditions.

Lipid competition experiments were carried out in 12 x 75 mm borosilicate glass tubes in a total volume of 150 µl binding buffer [25 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl2, 0.25 mM phenylmethylsulfonyl fluoride, 10 mM monothioglycolic acid, 30 µg/ml bacitracin, and 0.1% BSA (pH 7.4)] for 60 min at 24°C. Membrane homogenates were incubated in duplicate or in triplicate with approximately 0.15 nm [125I-Tyr]bombesin as radioligand and with increasing concentrations (10—12—10—6 M) of unlabeled [125I-Tyr]bombesin or structurally unrelated peptides as competitors. The reaction was terminated by adding 250 µl ice-cold binding buffer to the tubes, and the bound ligand was separated from free ligand by centrifugation at 4500 × g for 10 min at 4°C. The pellet was washed twice with 500 µl ice-cold buffer, and the radioactivity in the pellet of each tube was counted in a gamma counter (Micromedic Systems, Inc., Huntsville, AL). Some experiments were performed with membrane protein concentrations ranging from 10 to 100 µg/tube in order to determine the minimal amount of protein required to assess specific binding at a satisfactory level.

**Mathematical Analysis of the Binding Data.** Estimates of specific ligand binding capacities and affinities were calculated by the Ligand-PC computerized curve-fitting program of Munson and Rodbard (41) as modified by...
McPherson (42) and the Lundon-2 competition data analysis program (Lunden Software). To determine the types of receptor binding, dissociation constants ($K_d$) and the maximal binding capacity of receptors ($B_{max}$), bombesin binding data were also analyzed by the Scatchard method (36). Correlation coefficients ($r$) were calculated by a computer program. $P <0.05$ was accepted as a statistically significant difference.

RESULTS

Expression of Sex Hormone Receptors in Tumor Biopsies. To ensure that the population of human breast carcinomas utilized in this investigation of bombesin receptor expression was representative of both potentially endocrine-responsive and -unresponsive tumors, sex hormone receptors were examined in the cytosol preparations. Examination of estrogen receptor levels in biopsies as a function of patient age is presented in Fig. 1. Utilizing 50 years of age or younger for premenopausal status, the median estrogen receptor level was 45 fmol/mg cytosol protein ($n = 29$). The median estrogen receptor level was 98 fmol/mg for biopsy specimens from postmenopausal patients ($n = 71$).

Fig. 2 examines the relationship of patient age and progestin receptor levels in breast carcinomas. The median progestin receptor level was 17 fmol/mg for premenopausal patients ($n = 29$), while that for postmenopausal patients ($n = 71$) was 44 fmol/mg.

In general, progestin receptor level rises as a function of an increase in estrogen receptor level (e.g., 12). When this relationship was examined in the tumor population studied (Fig. 3), the equation of the line expressing the correlation was

$$y = 0.97x + 7.28$$

($r = 0.64$). Utilizing a value of ≤10 fmol/mg for estrogen receptor negativity, the median progestin receptor level was 5 fmol/mg ($n = 36$); estrogen receptor-positive tumors had a median progestin receptor level of 145 fmol/mg ($n = 64$). Utilizing a value of ≤10 fmol/mg for progestin receptor negativity, the median estrogen receptor level was 10 fmol/mg ($n = 47$); progestin receptor-positive tumors exhibited a median value of 172 fmol/mg estrogen receptors ($n = 53$).

Characterization of [Tyr$^4$]BN Receptors in Biopsies. Of the 100 tumor preparations examined, 27 contained sufficient membranes of high integrity resulting in Scatchard plots satisfactory for detailed analyses of specific [$^{125}$I]-Tyr$^4$BN binding. In six additional cases, limited quantities of tumor membrane fractions indicated the presence of [Tyr$^4$]BN receptors. Using a single saturating dose of ligand, 67 biopsies did not exhibit [Tyr$^4$]BN receptors regardless of the procedure applied. Using a computerized curve-fitting program, typical displacement curves were obtained. The nonlinear curve fitting and the Scatchard plot analyses suggested that the labeled [Tyr$^4$]BN was associated with two classes of receptor sites (Fig. 4). In 27 tumors analyzed by complete displacement, the high-affinity binding sites exhibited a mean dissociation constant ($K_d$) of $2.1 \pm 1.8$ (SD) nM (range, 0.1–7.8 nM) and a mean maximal binding capacity ($B_{max}$) of 237 ± 222 fmol/mg membrane protein (range, 12–1050 fmol/mg protein). The low affinity sites bound bombesin with a mean dissociation constant ($K_d$) of 0.3 ± 0.2 µM (range, 0.01–1.3 µM) and a mean maximal binding capacity ($B_{max}$) of 5.9 ± 5.5 pmol/mg membrane protein (range, 0.6–25 pmol/mg protein). In addition, more than 24% of BNR-positive samples displayed relatively elevated values (>100 fmol/mg membrane protein) of high-affinity [Tyr$^4$]BN-binding sites. From our experience with the expression of other peptide hormone receptors (4, 8, 14), levels greater than 100 fmol/mg membrane protein are "elevated" relative to those observed in nonmalignant breast tissue.
BOMBESIN/GRP RECEPTORS IN HUMAN BREAST CANCER

[187x400]0.05
[238x385]Kd1.1J3 M
[187x380]0.04 I@@z$@S@ t@sL/@S@
[238x372]W..03@
[188x365]0.03@ p@.I/_I p"I.
[205x296]0 500 1000 1500 2000
[242x285]BOUND(pM)
[329x134]Table 1 Distribution of bombesin receptors in human breast carcinomas
[329x134]sex hormone receptor statusaccording to
[336x134]to
[367x98]Bombesin receptors
[458x98]ER@PR@ER@PR@ER@PR@BNR@21
[470x86]363BNR29
[467x78]28 82

BOMBESIN/GRP RECEPTORS IN HUMAN BREAST CANCER [Tyr]

[1251

Tyr4

]BN Binding in Human Breast Cancer. A common error in receptor measurements arises from the instability of receptor binding at low protein concentrations. This point was assessed in two experiments in which human breast cancer membrane fractions prepared in homogenization buffer were serially diluted in the same buffer and assayed for [1251

Tyr4

]BN binding. This work indicates that accurate results can be obtained over a wide range (20—100 µg/tube) of membrane protein concentrations. For most assays, protein concentration in the incubates was maintained at approximately 20—50 µg/50 µL.

Correlation of Bombesin Receptors with Expression of Estrogen and Progestin Receptors. Table 1 and Figs. 6 and 7 show the

Table 1 Distribution of bombesin receptors in human breast carcinomas according to sex hormone receptor status

<table>
<thead>
<tr>
<th>Sex hormone receptor status</th>
<th>Bombesin receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER<em>PR</em></td>
<td>21</td>
</tr>
<tr>
<td>ER*PR</td>
<td>3</td>
</tr>
<tr>
<td>ER*PR</td>
<td>6</td>
</tr>
<tr>
<td>ER*PR</td>
<td>3</td>
</tr>
<tr>
<td>BNR*</td>
<td>29</td>
</tr>
<tr>
<td>BNR*</td>
<td>28</td>
</tr>
<tr>
<td>BNR*</td>
<td>8</td>
</tr>
<tr>
<td>BNR*</td>
<td>2</td>
</tr>
</tbody>
</table>

* ER and PR positivity, ≥10 fmol/mg cytosolic protein.

a BNR positivity, >10 fmol/mg membrane protein.
Table 2 Relationship between the binding capacities of [Tyr4]BN and those of ER and PR receptors in human breast cancer specimens

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[Tyr4]BN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of receptor</td>
<td>ER</td>
</tr>
<tr>
<td>ER</td>
<td>0.578</td>
</tr>
<tr>
<td></td>
<td>n = 50</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>PR</td>
<td>0.578</td>
</tr>
<tr>
<td></td>
<td>n = 50</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>[Tyr4]BN (high affinity)</td>
<td>0.671</td>
</tr>
<tr>
<td></td>
<td>n = 27</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>[Tyr4]BN (low affinity)</td>
<td>0.385</td>
</tr>
<tr>
<td></td>
<td>n = 23</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

a Correlation coefficient (r).

b n, number of human breast cancer specimens exhibiting specific binding to both receptors; NS, statistically insignificant correlation.

distribution of tumors containing [Tyr4]BN receptors as a function of estrogen and progesterone receptor status in 100 human breast cancer specimens examined. Tumor tissue samples containing BNR greater than 10 fmol/mg membrane protein were arbitrarily designated as positive while ER and PR values ≥10 fmol/mg cytosol protein were considered positive according to clinical response criteria established by the National Surgical Adjuvant Breast Project (37). Membrane preparations from 33 of 100 cases (33%) exhibited receptors for [Tyr4]BN. As shown in Table 1, ER was detected in 82% (27 of 33) and PR in 73% (24 of 33) of the tumors containing bombesin receptors with the characteristics of positivity defined above. Six of the 36 ER-negative tumors (17%) contained specific [Tyr4]BN receptors. Twenty-eight tumors did not contain significant levels of BNR, ER, or PR using the criteria described while 21 of 100 (21%) breast cancer specimens expressed all 3 receptors.

Previous reports have shown that expression of the sex steroid hormone receptors was positively associated (2, 4). In this study, when all sex steroid receptor values were considered, a statistically significant positive correlation was found between binding capacities of estrogen and progesterone receptors (r = 0.641; P < 0.001; n = 100) (Fig. 3). When only receptor values with at least 10 fmol/mg cytosol protein were considered, a statistically significant correlation (r = 0.578; P < 0.005; n = 50) (Table 2) was also found, indicating that ER and PR are not independent variables. To determine whether bombesin/GRP receptor concentrations were correlated with the expression of either ER or PR, the data were analyzed by a computer correlation test. A positive correlation, highly significant statistically, was shown to exist between [Tyr4]BN binding to low affinity sites and ER levels (Table 2). Although a positive correlation was established between concentrations of low affinity [Tyr4]BN-binding sites and PR-binding capacities (Table 2), there was no correlation between the concentrations of high-affinity binding sites of [Tyr4]BN and PR levels (Table 2). Fig. 6 illustrates the relationship between estrogen receptor expression in 100 tumor biopsies and BNR/GRP receptor content. Although most of the tumors did not express bombesin receptors, it is suggested that for those that do, the binding capacity increases as a function of estrogen receptor activity. The relationship...
between BN/GRP receptors and progestin-receptor expression is presented in Fig. 7. These data confirm the analyses shown in Table 2 that shows there was no correlation between the presence of high-affinity binding sites for [Tyr⁴]BN and PR levels. In addition, no correlation was observed between binding capacities of either high or low affinity [Tyr⁴]BN-binding sites (Table 2). Finally, no statistically significant correlation was found between the dissociation constants (K_d) of the two classes of [Tyr⁴]BN-binding sites in human breast cancer specimens examined (r = 0.115; n = 27).

**Relationship of Patient Age and [¹²⁵I-Tyr⁴]BN Binding.** The menopausal status of a patient is related to age. Most studies assume that 50 years of age is a useful time to evaluate marker expression as a function of premenopausal and postmenopausal status. There was no significant correlation of BNR levels with age of patients either when high-affinity [Tyr⁴]BN-binding sites were considered (r = 0.291; n = 27) or when low affinity [Tyr⁴]BN-binding sites were evaluated (r = 0.211; n = 27). As shown in Fig. 8 and Table 3, the presence of BNR in breast carcinomas was not related to patient age, with the exception that specific [Tyr⁴]BN-binding was not detected in biopsies from patients under 40 years of age. However, only 4 specimens were examined.

**DISCUSSION**

Bombesin, GRP, and related peptides influence the release of gastrointestinal hormones, stimulate gastric and pancreatic secretions, and gastrointestinal motility (19–21, 27, 29, 32). Bombesin-like immunoreactivity has been demonstrated in the brain, gastrointestinal tract, lungs, and mammary glands of various animals (19, 22). Bombesin and gastrin-releasing peptide also appear to function as autocrine or paracrine growth factors in a variety of cells (18, 19–23, 25, 26). These studies indicate that bombesin/GRP peptides are involved in the regulation of cell proliferation and differentiation. Our understanding of the biological effects of bombesin and GRP suggests that these peptides utilize membrane-bound receptors as part of their mechanism of signal transduction (20, 21, 24, 32).

As part of a long-term investigation of the relationship of peptide and steroid hormone receptors to human breast cancer progression and treatment, we characterized [Tyr⁴]BN receptors in biopsy samples of breast carcinomas. Several studies by our group (27, 31, 43, 44) using bombesin antagonists demonstrated the presence of bombesin/GRP receptors in human breast, pancreatic, and gastric cancer cells grown in tissue culture. Giacchetti et al. (23) also described BN/GRP receptors in certain breast cancer cell lines that were absent in normal breast epithelium. To our knowledge, this study is the first to identify and characterize these receptor proteins in biopsies of human breast carcinomas.

Examination of the binding of [¹²⁵I-Tyr⁴]BN was performed using membranes isolated from 100 human breast carcinomas. The analysis of complete displacement curves of [¹²⁵I-Tyr⁴]BN by unlabeled [Tyr⁴]BN and the Scatchard plots of these data indicate two classes of binding sites, one with high affinity (mean K_d = 2.1 nM) and low binding capacity (mean B_max = 237 fmol/mg membrane protein) and the other with low affinity (mean K_d = 0.3 μM) and high binding capacity (mean B_max = 5.9 pmol/mg membrane protein). The specificity of the receptors for [Tyr⁴]BN binding was studied using competitive ligand-binding experiments. These receptor proteins recognized bombesin and GRP(14–27) peptides in a specific manner with high affinity as ascertained by evaluation of a variety of unlabelled compounds. Since the affinity of [Tyr⁴]BN and GRP(14–27) was 100-fold higher than that of neurenomedin B, we concluded that human breast carcinomas contain primarily the BN/GRP receptor subtype. In addition, these data suggest that high-affinity binding represents association with bombesin/GRP receptors while low affinity binding represents association with neurenomedin B receptors. Further investigation using different ligands will distinguish the receptor subtypes. In contrast to human breast cancer cell lines, MDA-MB-231, MCF-7, MIII, and T-47D, in which a single class of high-affinity BN/GRP-binding sites were detected (23, 31), both high and low affinity binding sites were detected. All human breast cancer specimens, in our experience, demonstrated both high and low affinity binding sites, but the significance of these two types of sites is unclear at present. Thirty-three of 100 human breast carcinomas expressed [Tyr⁴]BN receptor levels of >10 fmol/mg membrane protein. The relatively wide variation in the dissociation constants and the maximal binding capacity of BN/GRP receptors is not clear at present. The presence of BN/GRP receptors of different affinity subtypes suggests that polymorphism may be related to biological response. Usually, the receptor isoform exhibiting the higher affinity has been correlated with clinical response to therapy as in the cases of estrogen and progestin receptors (7–9, 12) in breast cancer.

It is interesting to note that the expression of BN/GRP receptors appears to be related to clonal origin. Some human breast tumor cell

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Positive/total</th>
<th>% Positive</th>
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<tbody>
<tr>
<td>&lt;29</td>
<td>0/1</td>
<td>0</td>
</tr>
<tr>
<td>30–39</td>
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<td>9/21</td>
<td>43</td>
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<td>50–59</td>
<td>4/20</td>
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<tr>
<td>60–69</td>
<td>6/17</td>
<td>35</td>
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<tr>
<td>70–79</td>
<td>8/21</td>
<td>38</td>
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<tr>
<td>80–89</td>
<td>5/14</td>
<td>36</td>
</tr>
<tr>
<td>&gt;90</td>
<td>1/3</td>
<td>33</td>
</tr>
</tbody>
</table>

**Table 3** [¹²⁵I-Tyr⁴]bombesin-binding as a function of patient age

Human breast cancer specimens were assayed for specific [¹²⁵I-Tyr⁴]BN binding as described in "Materials and Methods." Tumor samples with 10 fmol or more of [¹²⁵I-Tyr⁴]BN bound specifically/mg of membrane protein were designated arbitrarily as BNR positive.

**Fig. 8.** Relationship of patient age and bombesin receptor expression in human breast carcinomas. Bombesin receptor levels were determined by ligand-binding assays as described in "Materials and Methods" using membrane preparations from breast tumor biopsies. Specific binding capacity is expressed as fmol/mg membrane protein.
A statistically significant correlation was also noted between the bombesin/GRP antagonists (45, 46). Since short-chain bombesin (6—14) of high-affinity [Tyr4]BN-binding sites and estrogen receptor levels. A highly significant positive correlation (P < 0.005) was observed between the binding capacities and estrogen receptor levels and between the concentrations of low affinity [Tyr4]BN-binding sites in MCF-7 cells and showed that bombesin stimulated inositol phospholipid hydrolysis and Ca2+ mobilization but did not stimulate proliferation. MCF-7 MIII subline isolated from MCF-7 tumors proliferating in nude ovariectomized mice also showed high-affinity bombesin receptors (31).

The duration of remission of breast cancer produced by treatment with antiestrogens and endocrine ablation (ovariectomy) is varied, and many hormone-responsive tumors become unresponsive (7, 10). The presence of both estrogen and progesterin receptors in a breast cancer biopsy indicates a 75—80% likelihood that the patient will respond to endocrine manipulations (7, 8, 12). Other patients, usually those with receptor-negative tumors, do not benefit from antiestrogen therapy. New approaches should be explored to improve therapy for endocrine-independent tumors which represent a large number of breast cancers in women. The discovery of bombesin/GRP-like peptides functioning as autocrine growth factors in human small cell lung carcinoma (18, 20) stimulated the development of several classes of bombesin/GRP antagonists (45, 46). Since short-chain bombesin (6—14) antagonists with a reduced peptide bond between positions 13 and 14 were reported to be 100 times more potent than earlier antagonists (45), many modified pseudononapeptide-bombesin antagonists were synthesized and tested for inhibition in our laboratory (27, 30, 31, 43, 46). RC-3095 was one of the most powerful of these antagonists (46).

Recently, we reported that bombesin stimulated the proliferation of MCF-7 MIII and estrogen-independent MDA-MB-231 human breast cancer cell lines in vitro and that RC-3095 inhibited this growth as measured by the decrease in cell number and the reduction in [3H]thymidine incorporation into DNA (27). The growth inhibition of estrogen-dependent and -independent MXT mammary cancer in mice by the BN/GRP antagonist RC-3093 (30) and the suppressive effect of this peptide on growth of MCF-7 MIII human breast cancer xenografts in athymic nude mice were also reported (31).

A highly significant positive correlation (P < 0.005) was observed between the binding capacity of high-affinity [Tyr4]BN-binding sites and estrogen receptor levels and between the concentrations of low affinity [Tyr4]BN-binding sites and progestin receptor levels (P < 0.05). It is accepted that the expression of estrogen and progestin receptors in breast carcinoma biopsies is correlated with a greater incidence of response to endocrine therapy (8—10). Furthermore, the presence of these sex hormone receptors in a tumor biopsy is related to improved prognosis (7). Because sex hormone receptor expression is associated with a potential endocrine response in breast cancers, we analyzed the presence of BN/GRP receptors in different tumor subtypes. When the levels of BN/GRP receptors were compared to the content of the sex steroid receptors, a highly significant positive correlation (P < 0.005) was observed between the binding capacities of high-affinity [Tyr4]BN-binding sites and estrogen receptor levels. A statistically significant correlation was also noted between the concentrations of low affinity [Tyr4]BN-binding sites and progestin receptor levels (P < 0.05), although at a lower level of confidence.

To our knowledge, this is the first report of these regulatory proteins in human breast carcinomas. Expression of specific receptor proteins for BN/GRP in a large number of human breast cancers suggests that these potent mitogens may be involved in tumor cell progression. Measurement of bombesin/GRP receptors may provide an additional means of characterizing human breast cancers with respect to their metastatic potential, as has been suggested for EGF receptors (3, 4, 11). Furthermore, the identification of these receptors directly on membranes of human breast carcinomas provides a greater stimulus for the development of BN/GRP antagonists with therapeutic potential. It is also possible that, on the basis of such receptor studies, highly active bombesin/GRP antagonists could be used for the treatment of selected breast cancers in women.

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Characterization of Bombesin/Gastrin-releasing Peptide Receptors in Human Breast Cancer and Their Relationship to Steroid Receptor Expression

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