Insulin Receptor Expression and Function in Human Breast Cancer Cell Lines

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ABSTRACT

We have previously reported that insulin receptor expression is increased in human breast cancer specimens (V. Papa et al., J. Clin. Endocrine Research, Mount Zion Medical Center of the University of California, San Francisco, California 94120 [M. R. S.]). In the present study, in order to further understand the role of the insulin receptor in breast cancer, insulin receptor expression and function were characterized in three human breast cancer cell lines, MCF-7, ZR-75-1, and T-47D, and compared to a nonmalignant human breast epithelial cell line, 184B5. Insulin receptor content, measured by radioimmunoassay, was elevated 5- and 3-fold in MCF-7 and ZR-75-1 breast cancer cell lines, respectively, when compared to the nonmalignant cell line 184B5. In contrast, the insulin receptor content of T-47D cells was not increased. The increase in insulin receptor content in MCF-7 and ZR-75-1 cells was not due to amplification of the insulin receptor gene. Also, total insulin receptor mRNA content was not increased in breast cancer cells in respect to nonmalignantly transformed 184B5 breast epithelial cells. However, significant differences in the content of receptor mRNA species were observed.

The insulin receptors in the breast cancer cell lines were functional: (a) In all 4 cell lines, high-affinity insulin-binding sites were detected, and, in concert with the insulin receptor radioimmunoassay data, binding capacity was highest in MCF-7 and then in ZR-75-1 cells. (b) In all cell lines, insulin stimulated insulin receptor tyrosine kinase activity. However, the effect of insulin was greatest in breast cancer cell lines than in nonmalignant breast cells. (c) In all cell lines, insulin at concentrations of 1 nM or less stimulated [3H]thymidine incorporation. This effect of insulin was inhibited by 50% in MCF-7 cells and by 60% in 184B5 cells when α-IR3, a monoclonal antibody to the insulin-like growth factor I receptor, was present. (d) In these cells, therefore, insulin was active via both its own receptor and the IGF-I receptor. In contrast, α-IR3 antibody was without effect in T-47D and ZR-75-1 cells, suggesting that in these cell lines insulin acted only via its receptor. In the breast cancer cells, MA-5, an agonist monoclonal antibody to the insulin receptor, stimulated [3H]thymidine incorporation. This present study indicates therefore that in breast cancer cell lines there are functional insulin receptors that regulate breast cancer cell growth.

INTRODUCTION

Growth factor receptors of the tyrosine kinase family play a key role in both normal and neoplastic cell growth. The insulin receptor belongs to the tyrosine-kinase growth factor receptor family (1–3), and insulin mediates proliferative responses in a variety of both normal and transformed cells (4, 5). However, the role of the insulin receptor molecule in human neoplasia has not yet been established. Recently, we have reported that overexpression of the insulin receptor is a characteristic feature of many human breast cancer specimens (6). By using a specific insulin receptor radioimmunoassay, we found that the average insulin receptor content of human breast cancer specimens was approximately 6-fold higher than that of normal breast tissue. With immunohistochemical analysis we localized the increased expression of the insulin receptor to the malignant epithelial cells. The insulin receptor content of the breast cancer specimens was positively correlated with tumor grade and tumor size. Moreover, we have recently demonstrated that with fibroblasts and ovary cells transfected with and overexpressing insulin receptors, the addition of insulin induced a ligand-dependent transformed phenotype (7). These observations suggested, therefore, a possible role for insulin receptor overexpression in human cancer initiation and/or progression.

Human breast cancer cells in tissue culture have been important in vitro models for studying the regulation of breast cancer tissue by hormones and growth factors. Prior studies have identified the presence of insulin receptors in breast cancer cell lines (8). In certain human breast cancer cell lines insulin stimulates several cellular functions including cell growth (9, 10), and recently we have reported that progestins enhance the mitogenic effects of insulin (11). However, it is unknown whether insulin receptors are overexpressed in cultured human breast cancer cells and if the insulin receptor plays a role in the growth regulation of these cells. In the present study we have quantitated insulin receptor content in 3 lines of human breast cancer cells in tissue culture. In addition, in these cell lines, we have studied insulin receptor tyrosine kinase activity and the ability of insulin to stimulate cell growth via its own receptor.

MATERIALS AND METHODS

Materials

The following materials were purchased: BSA (radioimmunoassay grade); bacitracin; phenylmethylsulfonylfluoride; Triton X-100; poly(Glu-Tyr); hybridization solution; sonicated salmon sperm DNA; dextran sulfate; formamide; N-acetyl-D-glucosamine; wheat germ agglutinin-agarose; and porcine insulin were from Sigma Chemical Company (St. Louis, MO). PVC plates were from Becton Dickinson Labware (Oxnard, CA). [125I]-Labeled Bolton-Hunter reagent (2200 Ci/mmol), [α-32P]ATP (3000 Ci/mmol), and [α-32P]dTCTP (3000 Ci/mmol) were obtained from Amersham International (Amersham, England). [3H]Insulin (specific activity, 2200 Ci/mmol) and [3H]thymidine (82.3 Ci/mmol) were from New England Nuclear (Boston, MA).

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2 To whom requests for reprints should be addressed, at Division of Diabetes and Endocrine Research, Mount Zion Medical Center, P. O. Box 7921, San Francisco, CA 94120.

The abbreviations used are: BSA, bovine serum albumin; PVC, polyvinylchloride; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; poly(A) +, polyadenylated; IGF-I, insulin-like growth factor I; poly(Glu-Tyr), polymer of glutamic acid and tyrosine.

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Cell Culture
MCF-7, T-47D, and ZR-75-1 human breast cancer cell lines (from Dr. I. Perroteau, Turin, Italy) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, glutamine, non-essential amino acids, penicillin, and streptomycin (Cell Culture Facility, University of California, San Francisco, CA). 184 nonimmortalized breast epithelial cells and 184B5 breast epithelial cells, which are an immortalized but not malignantly transformed human breast cell line (12), were cultured in MCDB-170 media supplemented with bovine pituitary extracts (Clonetic Corporation, San Diego, CA) and other factors as previously indicated (12). Insulin receptor mononclonal antibodies were prepared as previously described (13). α-IR3 was from Oncogene Sciences, Inc. (Cambridge, MA).

Insulin Receptor Content in Cancer Cell Lines

Evaluation by Radioimmunoassay. To prepare extracts for insulin receptor radioimmunoassay, cell monolayers were preincubated for 24 h in serum-free medium containing 0.1% BSA and 10 μg/ml transferrin and then harvested with phosphate-buffered saline and 0.2% EDTA, and the cells were counted in a Neubauer chamber. Cells (3 x 10⁶) were solubilized in 1 ml of 50 mM HEPES buffer, pH 7.4, containing 2 mg/ml bacitracin, 1 mM phenylmethylsulfonylfluoride, and 1% Triton X-100 for 60 min at 4°C under continuous shaking. The solubilized material was then centrifuged at 10,000 x g, and the supernatant was frozen at -80°C until assayed. The DNA content in the cellular extracts was measured by the method of Labarca and Paigen (14).

The insulin receptor radioimmunoassay was performed as previously described (15) using pure placental insulin receptors for a standard (16), labeling them with 125I-Bolton Hunter reagent, and using an anti-insulin receptor polyclonal antiserum (final dilution, 1:100,000). This antiserum reacts with epitopes in both the α and β subunits of the receptor (15). Bound/free radioactivity separation was obtained by precipitation with goat anti-rabbit antiserum. In this assay cross-reactivity with the related IGF-I receptor was less than 1%.

Evaluation by 125I-Insulin Binding to Intact Cells. 125I-Insulin binding studies were carried out in intact cells grown to confluent monolayers in tissue culture flasks. Cells were preincubated for 24 h in serum-free medium containing 0.1% BSA and 10 μg/ml transferrin and then harvested as described previously, washed twice with phosphate-buffered saline, and then resuspended in the binding buffer (50 mM HEPES, pH 7.8, 120 mM NaCl, 1.2 mM MgSO₄, 5 mM KCl, 15 mM sodium acetate, 10 mM glucose, 1 mM EDTA, 10 mg/ml BSA, and 1 mg/ml bacitracin) at a final concentration of 3 x 10⁶ cells/ml. Binding assays were carried out in 12 x 75 mm borosilicate tubes for 16 h at 4°C (0.5 ml final volume with 40 μM labeled insulin) without or with increasing concentrations of unlabeled insulin. At the end of the incubation, 1 ml of binding buffer at 4°C was added, and the tubes were centrifuged for 10 min at 2400 rpm. Cell pellets were then washed twice and lysed with 0.03% SDS, and the radioactivity was counted in a gamma counter. Nonspecific binding was 0.4-0.6% of total radioactivity and was subtracted. Scatchard plots were resolved into two orders of binding sites using the computer software ENZ-FITTER.

Insulin Receptor Kinase Studies
Confluent monolayers of cell cultures were harvested, solubilized, and centrifuged as described above. The soluable material was immediately applied to a 1-mL wheat germ agglutinin agarose column pre-equilibrated with column buffer containing 150 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonylfuoride, 2 mg/ml bacitracin, and 50 mM HEPES, pH 7.6. Glycoproteins were eluted with the same buffer containing 0.3 M N-acetyl-d-glucosamine. The concentration of the insulin receptor in each fraction was assessed by radioimmunoassay (15).

The same amount of insulin receptors from each cell line (10 ng each) was assayed for tyrosine kinase activity toward the exogenous substrate poly(Glu-Tyr) using a specific PVC plate assay (17). The wells of PVC plates were first coated with 50 μl of rabbit anti-mouse IgG (40 μg/ml) at 4°C for 16 h. After 4 washes with washing buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1% Tween 20, 0.1% BSA), the plates were incubated at 4°C for 16 h with 10 μg/ml of a specific monoclonal antibody to the insulin receptor MA-20 (13). (This antibody does not have insulin agonist effects in breast cancer cells.) This step was followed by further incubation with normal mouse IgG (150 μg/ml) at 4°C for 16 h. Ten ng of insulin receptors, prepared from various cell lines, were then added to the plates and incubated at 4°C for 16 h. After 4 washes with washing buffer, 24 μl of a reaction mixture containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 2 mM MnCl₂, 10 mM MgCl₂, and various concentrations of insulin were added into each well and incubated at 22°C for 1 h. The kinase reaction was started by the addition of 3 μl of poly(Glu-Tyr) (2 mg/ml) and 10 μl of [γ-32P]ATP (1 μCi/well).

After 1 h at 22°C, 10 μl of the reaction mixture were spotted on 3 MM filter paper and precipitated in cold 10% trichloroacetic acid followed by scintillation counting.

Cell Growth Experiments in Monolayer Cultures

[3H]Thymidine Incorporation into DNA. Cells (40 x 10⁶) were plated in 24-well tissue culture plates in their regular growth medium. After 48 h, the medium was removed and replaced by fresh medium containing 0.1% BSA and 10 μg/ml transferrin, except in T-47D cells, where Dulbecco’s modified Eagle’s medium supplemented with 5% charcoal-stripped fetal calf serum and 10 μM progesterone was used (11). Seventy-two h later, variable concentrations of either insulin, MA-5, or α-IR3 were added in fresh medium. After 24 h 0.5 μCi/well of [3H]thymidine was added for 2 h. Cells were then harvested, and the rate of DNA synthesis was measured as described previously (18).

We also measured DNA synthesis by direct assay of DNA. For this assay, 5 x 10⁴ cells were seeded in 35-mm multiwell plates and cultured as previously described. After 48 h, cells were washed twice with 1 ml of serum-free medium, and 2 ml of fresh serum-free medium were added. After 24 h insulin was added at the indicated concentrations. Insulin was then added every other day. Medium was changed every 2 days. At the end of a 5-day period the cells were solubilized in 0.03% SDS, and DNA content was determined (14).

DNA and RNA Analysis
Poly(A)⁺ RNA was extracted from cell monolayers (typically 10⁸ cells) using a new, one-step method as previously described (19). Briefly, adherent cells were released with protease K (final concentration, 0.3 mg/ml) and solubilized in 1% sodium dodecyl sulfate. Oligo(deoxythymidine)-cellulose was directly added to the lysate and incubated overnight at 22°C. Poly(A)⁺ RNA was eluted from oligo(deoxythymidine)-cellulose by adding 3 ml of 10 mM Tris with 0.1 mM EDTA and 0.2% SDS. Poly(A)⁺ RNA (8 μg) was then electrophoresed on 1% agarose gel containing 2% formaldehyde and then transferred to nitrocellulose filters. Poly(A)⁺ RNA content was normalized by using an oligo(deoxythymidine) probe that was end-labeled using the enzyme T4 polynucleotide kinase (20).

High-molecular-weight DNA from cell pellets was extracted by the proteinase K-form method, resuspended in a buffer containing 100 mM NaCl, 10 mM Tris, and 10 mM EDTA at pH 8.0, and dissolved by the addition of 0.5% SDS in the presence of 100 μg/ml of protease K at 37°C. DNA was digested with the restriction endonuclease EcoRI under standard conditions. Twenty μg of the digested DNA were subjected to electrophoresis on 0.8% agarose gel, followed by denaturation in NaOH buffer and immobilization to a nitrocellulose filter as described by Southern (21). For slot blot experiments nucleic acids were applied to nitrocellulose paper using a Slot-Blot Minifold apparatus (Schleicher and Schuell, Keene, NH).

Northern and Southern blot hybridizations were carried out using two human insulin receptor cDNA probes, 18.2 and 13.2 (1 and 4.2 kilobases, respectively), a kind gift of Dr. G. I. Bell (University of Chicago). These were labeled with 100 μCi [γ-32P]CTP by random primers (20) to a specific activity of 10⁶ cpm/μg. The nitrocellulose
filters from both Northern and Southern blots were prehybridized, hybridized, and washed as previously described (21).

RESULTS
Insulin Receptor Expression

Insulin Receptor Radioimmunoassay. In order to determine the insulin receptor content in the breast cancer lines studied, cells were solubilized and the insulin receptor content measured by a specific insulin receptor radioimmunoassay. Increasing amounts of cellular extracts produced competition-inhibition curves that were parallel to the highly purified insulin receptor standard (Fig. 1). The content of receptors varied from 28.5 ng/10⁶ cells in the MCF-7 cells to 4.8 ng/10⁶ cells in the T-47D cells (Table 1). In the MCF-7 and ZR-75-1 cell lines, the insulin receptor content was considerably higher than in 184B5 cells [5.3 ± 1.7 (SEM) ng/10⁶ cells], a nonmalignantly transformed human breast epithelial cell line. The insulin receptor content in T-47D cells (4.8 ± 1.2 ng/10⁶ cells) was similar to that in 184B5 cells. The nonimmortalized parent cell of 184B5 cells, 184 cells, was also analyzed and had a similar insulin receptor content (4.1 ± 0.2 ng/10⁶ cells).

Insulin Receptor Gene Content. To ascertain whether gene amplification may have occurred in breast cancer cell lines, Southern blot analyses were carried out (Fig. 2). DNA was isolated and digested with the restriction endonuclease EcoRI and hybridized with labeled insulin receptor cDNA. No evidence of gene amplification was seen with DNA from MCF-7, ZR-75-1, and T-47D cells when compared with DNA from normal breast tissue. Slot blot hybridization was also carried out with both insulin receptor and β-actin probes (Fig. 3). Densitometric analyses were carried out, and the ratio of insulin receptor DNA to actin DNA was the same.

Insulin Receptor mRNA Content. In order to assess whether the differences in the insulin receptor protein content were associated with differences in insulin receptor mRNA levels, poly(A)^+ RNA from the breast cancer cell lines and the nonmalignant breast epithelial cell line were subjected to Northern blot analysis (Fig. 4). In all cell lines except T-47D cells, prominent bands of insulin receptor mRNA were seen at 11.0 and 8.5 kilobases, although the ratio between the two bands varied in the different cell lines (Fig. 5). In T-47D cells only the 8.5-kilobase band was prominent. Except for T-47D cells, which had reduced insulin receptor mRNA, there was no major quantitative difference in insulin receptor mRNA content.

Insulin Receptor Functional Studies

Ligand Binding. Studies were next carried out to determine the functional properties of the insulin receptors in the breast cancer cell lines and the nontransformed breast epithelial cells. First, in intact cells the α subunit function of the insulin receptor was investigated with ligand binding studies (Fig. 6) and analyzed by Scatchard analysis (22). In all cell lines, binding was a curvilinear function, compatible with the presence of two orders of binding sites (23), a low-affinity–high-capacity site and a high-affinity–low-capacity site (Table 2). Each cell line had different binding characteristics (Table 2). Major differences were seen in binding capacity. MCF-7 cells had the highest total binding capacity (high- and low-affinity sites) with a value of 433 fmol/3 × 10⁶ cells, whereas T-47D cells had the
Insulin induced a dose-dependent increase of [3H]thymidine incorporation in all the cell lines (Fig. 8). In these cell lines insulin was effective at concentrations of 0.1–1.0 nm. Maximal responsiveness, however, was over 2-fold greater in MCF-7 cells than in other cells. Other studies revealed similar results when DNA content was measured after 5 days of incubation with insulin (Table 3).

To evaluate potential insulin signaling via the IGF-I receptor, we next investigated the effect of α-IR3 (24), a monoclonal antibody that inhibits binding to the IGF-1 receptor, on insulin-stimulated [3H]thymidine incorporation (Fig. 9). α-IR3 inhibited 50–60% of the insulin effect in MCF-7 and 184B5 cells. In contrast, this antibody did not inhibit [3H]thymidine incorporation in ZR-75-1 and T-47D cells. Normal mouse IgG (100 nM) produced only a slight inhibition (10%) of the insulin effect (not shown).

Next we stimulated these cells with MA-5, an insulin agonist monoclonal antibody to the insulin receptor (25). MA-5 stimulated [3H]thymidine incorporation in all cell lines studied. The lowest capacity of 58 fmol/3 x 10⁶ cells. The affinities of both classes of binding sites were relatively higher in ZR-75-1 and T-47D cells than in MCF-7 and 184B5 cells.

Insulin Receptor Kinase. We next measured the tyrosine kinase activity of the insulin receptors from the cell lines. For this purpose we used a sensitive and specific plate assay that captures the insulin receptor with an insulin receptor monoclonal antibody (17). The assay measures insulin receptor tyrosine kinase activity and not other tyrosine kinases (17). Insulin receptors were extracted from all cell lines and normalized to 10 ng of insulin receptor by radioimmunoassay. In all cells insulin via the insulin receptor stimulated the phosphorylation of poly-(Glu-Tyr). Interestingly, the insulin-stimulated receptor tyrosine kinase activity in all breast cancer cells was 5–10-fold higher than in nonmalignant cells. The greatest increase in insulin receptor tyrosine kinase activity was observed in ZR-75-1 cells (Fig. 7).

Insulin Effect on Cell Growth

In order to investigate the mitogenic effect of insulin on these cell lines we studied [3H]thymidine incorporation into DNA.
of these multiple mRNA species are involved in insulin receptor was observed only after progesterone treatment (11). In most ence was observed in the specific mRNA content of MCF-7 and protein synthesis, since all are found to be associated with ri-

Thus cannot account for such size heterogeneity. Most likely, all start sites, but they differ by only several hundred bases (27) and of the insulin receptor RNA (3, 26). There are also multiple 5' only the 8.5-kilobase band was prominent; the 11-kilobase band from 5.2 to 11 kilobases (3). Studies of these multiple transcripts suggest that they are due to variable splicing at the 3' end human and animal cells that have been studied, several insulin receptor mRNA species are observed, with a size ranging widely from 1.16 to 23.77 ng/0.1 mg protein, but the mean value, 6.15 ng/0.1 mg protein, was 6-fold higher than the mean value found in normal breast tissues, 0.96 ng/0.1 mg protein. The insulin receptor mean content in human breast cancer specimens was also higher than that observed in any normal human tissue, including liver. Immunostaining analysis revealed that the insulin receptor content of breast cancer specimens was due to an increased receptor content of the malignant breast epithelial cells. We also documented that the insulin receptors in breast cancer specimens were functional: insulin binding was increased in proportion to insulin receptor content as measured by radioimmunoassay, and insulin receptor kinase activity was present and responsive to insulin.

In the present study, we found that insulin receptor expres-

sion in human cultured breast cancer cells in vitro was variable, as it was in the breast cancer specimens in vivo. In two of the three cancer cell lines studied (MCF-7 and ZR-75-1), insulin receptor expression was increased 6- and 3-fold relative to both the nonmalignantly transformed human breast cells, 184B5, and their nonimmortalized parent 184 cells. Analysis of the insulin receptor gene indicated that the increased insulin receptor content in MCF-7 and ZR-75-1 cells was not due to gene amplification, since the different cell lines examined. When insulin receptor mRNA was studied, no major difference was observed in the specific mRNA content of MCF-7 and ZR-75-1 cells with respect to the nonmalignantly transformed breast cell line 184B5. In the malignant (MCF-7 and ZR-75-1) and nonmalignant (184B5) cells, both the 8.5- and 11-kilobase insulin receptor mRNA bands were prominent even if the ratio of the two bands varied. As previously reported, in T47-D cells only the 8.5-kilobase band was prominent; the 11-kilobase band was observed only after progesterone treatment (11). In most human and animal cells that have been studied, several insulin receptor mRNA species are observed, with a size ranging widely from 5.2 to 11 kilobases (3). Studies of these multiple transcripts suggest that they are due to variable splicing at the 3' end of the insulin receptor RNA (3, 26). There are also multiple 5' start sites, but they differ by only several hundred bases (27) and thus cannot account for such size heterogeneity. Most likely, all of these multiple mRNA species are involved in insulin receptor protein synthesis, since all are found to be associated with ri-

bosomes (28). However, the biological role for these multiple transcriptions is unknown, and, therefore, their possible role in the enhanced insulin receptor expression in human breast cancer cells cannot be excluded. In addition to the observed changes in mRNA quality, an enhanced translation of the insulin receptor mRNA or an enhanced stability of the insulin receptor protein may also be responsible for the relatively high insulin receptor content in human breast cancer cells.

Insulin receptors were functional in the different cell lines. In agreement with insulin receptor content as measured by radio-

immunoassay, insulin binding capacity was highest in MCF-7 and ZR-75-1 cells and lowest in T-47D and 184B5 cells. The higher receptor affinity observed in ZR-75-1 and T-47D cells could be due to changes in either posttranslational modification of insulin receptor or the plasma membrane environment (3).

We also studied the function of the insulin receptor ß subunit with a tyrosine kinase plate assay (17) which separates insulin receptor kinase activity from the kinase activities of other receptors, including the closely related IGF-1 receptor. Using this assay, with insulin receptors from all cell lines, we found that insulin stimulated tyrosine phosphorylation of the substrate poly(Glu-Tyr). Insulin-stimulated insulin receptor tyrosine kinase activity was higher in MCF-7 and ZR-75-1 cells than in T-47D and 184 B5 cells. This observation suggests that heter-

ogeneity exists in insulin receptors in the different cell lines. This observation is not surprising, since heterogeneity of insulin receptor ß subunits exists between various human tissues (29). One explanation for this heterogeneity is differences in the glycosylation pattern of the insulin receptors.

A mitogenic effect of insulin was observed in all breast cancer cell lines, insulin being effective at physiological concentrations of 1 nm or less. When we used a-IR3, an IGF-1 receptor-blocking antibody, it was without effect on insulin-stimulated [3H]-thymidine incorporation in T-47D and ZR-75-1 cells. In con-

trast, it partially inhibited the insulin effect on this function in MCF-7 and 184B5 cells. This study suggested therefore that in

### Table 2 Characteristics of high- and low-affinity insulin binding to cultured breast cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>KD (nm)</th>
<th>Binding capacity (fmoles/3 x 10^6 cells)</th>
<th>KD (nm)</th>
<th>Binding capacity (fmoles/3 x 10^6 cells)</th>
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<td>MCF-7</td>
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<td>9</td>
<td>375</td>
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<tr>
<td>ZR-75-1</td>
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<td>2.1</td>
<td>160</td>
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<tr>
<td>T-47D</td>
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<td>3</td>
<td>47</td>
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<tr>
<td>184B5</td>
<td>0.6</td>
<td>26</td>
<td>5.2</td>
<td>128</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Previously we studied insulin receptors in 159 human breast cancer specimens (6). The expression of these receptors was heterogeneous. The insulin receptor content in these specimens ranged from 1.16 to 23.77 ng/0.1 mg protein, but the mean value, 6.15 ng/0.1 mg protein, was 6-fold higher than the mean value found in normal breast tissues, 0.96 ng/0.1 mg protein. The insulin receptor mean content in human breast cancer specimens was also higher than that observed in any normal human tissue, including liver. Immunostaining analysis revealed that the insulin receptor content of breast cancer specimens was due to an increased receptor content of the malignant breast epithelial cells. We also documented that the insulin receptors in breast cancer specimens were functional: insulin binding was increased in proportion to insulin receptor content as measured by radioimmunoassay, and insulin receptor kinase activity was present and responsive to insulin.

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![Fig. 7. Phosphotransferase activities of the semipure insulin receptors in vitro. Semipure insulin receptors from ZR-75-1, MCF-7, T47D, and 184B5 cells were prepared by wheat germ agglutinin chromatography. Insulin receptors (10 ng based on radioimmunoassay) were then preincubated with various concentrations of insulin in PVC plates preabsorbed with the specific anti-insulin receptor monoclonal antibody MA-20. The reaction was started by the addition of an exogenous substrate, poly(Glu-Tyr), and 1 μCi [γ-32P]ATP. The radioactivity incorporated into poly(Glu, Tyr) was then measured by liquid scintillation. The data are expressed as pmol of ATP incorporated into 1 mg of poly(Glu, Tyr). A representative of two separate experiments is shown. Data are mean ± SD of quadruplicate determinations.](https://cancerres.aacrjournals.org/figure/fig7/)
INSULIN RECEPTOR IN HUMAN BREAST CANCER

Fig. 8. Stimulation of \([\text{^3}H]\)thymidine incorporation by insulin and insulin receptor agonist monoclonal antibody MA-5 in human breast epithelial cells and human breast cancer cells. Cells were plated in tissue culture plates in their regular growth medium. After 48 h medium was replaced with serum-free medium except for T-47D, for which 5% charcoal-stripped fetal calf serum and \(10^{-8}\) M progesterone-containing medium was used. After a further 72 h the cells were stimulated with either insulin or MA-5 for 24 h. Thymidine incorporation was performed during the last 2 h of stimulation by adding 0.5 nCi \([\text{^3}H]\)thymidine. Cells were then harvested, and the rate of \([\text{^3}H]\)thymidine incorporation was determined as previously described (12). Normal mouse IgG at 100 nM produced slight stimulation (5-10%) of \([\text{^3}H]\)thymidine incorporation. Each value is the mean ± SD of two separate experiments performed in triplicate.

MCF-7 and 184B5 cells, the insulin mitogenic effect was mediated partially via the insulin receptors and partially via the IGF-I receptors, whereas in T-47D and ZR-75-1 cells, insulin acted only via its own receptors. Moreover, MA-5, an anti-insulin receptor-specific monoclonal antibody and an insulin agonist, stimulated DNA synthesis in all cells. The mitogenic response to insulin receptor stimulation by MA-5 was greater in the breast cancer cells than in the nonmalignant breast cells. These studies strongly suggested a role for insulin receptor in the growth regulation of breast cancer cells. Moreover, the greater mitogenic effect of insulin via the insulin receptor in breast cancer cells when compared to nonmalignant breast cells is in concert with the observation that breast cancer cells have greater insulin-stimulated receptor tyrosine kinase activity. Interestingly, in MCF-7 cells Cullen et al. (30) did not observe inhibition by α-IR3 of the insulin mitogenic effect.

The biological significance of increased insulin receptor expression in breast cancer epithelial cells is unknown. Human and animal breast carcinomas have receptors for steroid and peptide hormones, and both in vitro and in vivo studies indicate that their growth is at least partially hormone dependent (31–35). Various studies have demonstrated that breast carcinomas containing high concentrations of estrogen and progesterone receptors have a better prognosis. However, some patients with breast carcinomas containing high concentrations of estrogen and progesterone receptors do not show a beneficial response to

<table>
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<th>Cells</th>
<th>Insulin (nM)</th>
<th>MCF-7</th>
<th>T-47D</th>
<th>ZR-75-1</th>
<th>184B5</th>
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<tr>
<td>0</td>
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<td>34.6±4</td>
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<td>34.6±2.0</td>
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<tr>
<td>1.0</td>
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<td>41.5±5</td>
<td>39.6±4</td>
<td>46.2±8</td>
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<tr>
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<td>46.6±7</td>
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<td>60.0±10</td>
<td>63.0±6</td>
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</tr>
</tbody>
</table>
INSULIN RECEPTOR IN HUMAN BREAST CANCER

hormonal therapy (36). One possible explanation for this discrepancy is that other classes of hormones such as polypeptide hormones and growth factors, either by themselves or in combination with steroid hormones, may stimulate tumor growth (31, 37). Insulin regulates the growth and metabolism of animal breast cancer cells both in vivo and in vitro (38-40) and human breast cancer cells in vitro (9, 10). It is likely, therefore, that in some breast cancers insulin, either alone or in combination with steroid and other related hormones and growth factors (41-42), plays a role in promoting their growth.

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Insulin Receptor Expression and Function in Human Breast Cancer Cell Lines

Giovanni Milazzo, Francesco Giorgino, Giuseppe Damante, et al.


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