Insulin and Insulin-like Growth Factor-I (IGF-I) Receptor Overexpression in Breast Cancers Leads to Insulin/IGF-I Hybrid Receptor Overexpression: Evidence for a Second Mechanism of IGF-I Signaling

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ABSTRACT

The insulin receptor (IR) form hybrids with the closely related insulin-like growth factor-I (IGF-I) receptor (IGF-I-R). Because most human breast carcinomas overexpress both the IR and the IGF-I-R, we evaluated whether the insulin/IGF-I hybrid receptor (Hybrid-R) is also overexpressed in these tumors and what role it plays in breast cancer biology.

Using specific ELISAs and Western blots, we measured Hybrid-R content and function in 8 human cultured breast cancer cell lines and 39 human breast cancer specimens. Hybrid-R content and function were also compared to the content and function of the IR and the IGF-I-R. Hybrid-R content exceeded the IGF-I-R content in >75% of breast cancer specimens and was directly related to the molar ratio of both the IR and IGF-I-R content, suggesting that Hybrid-R formation occurred by random assembly of IR and IGF-I-R half-receptors. Hybrid-Rs became tyrosine auto-phosphorylated when breast cancer cells were exposed to IGF-I but not when they were exposed to insulin.

In cells with an elevated Hybrid-R content, Hybrid-R autophosphorylation in response to IGF-I exceeded IGF-I-R autophosphorylation, suggesting that most of the IGF-I effect occurred via the Hybrid-R. Furthermore, Hybrid-Rs mediated growth in response to IGF-I, as indicated by experiments with blocking antibodies to the IGF-I-R.

These data indicated therefore that: (a) Hybrid-Rs are present and play a major role in mediating the IGF-I signal in breast cancer; (b) their expression is directly related to IR overexpression; and (c) potential therapies designed to block IGF-I actions in breast cancer must take into account the role of these Hybrid-Rs.

INTRODUCTION

Both the IR and the closely related IGF-I-R are overexpressed in most human breast cancer specimens and cultured cells (1, 2), and both play a role in the biology of breast cancer (3, 4). When overexpressed, both the IR and the IGF-I-R induce a ligand-dependent transformed phenotype (5, 6), and their content in breast cancer correlates with clinical outcome (2, 7–9). The ligand for IGF-I-R, IGF-I, is produced by stromal fibroblasts in the cancer tissue and acts in a paracrine manner on the IGF-I-R of breast cancer cells (10, 11). In contrast, insulin, the ligand for the IR, is not locally produced. Thus, the exact role and hormone regulation of the IR in breast cancer are unknown.

Both the IR and the IGF-I-R are tetrameric complexes consisting of two identical extracellular α-subunits that bind the hormone and two identical β-subunits that have ligand-activated tyrosine kinase activity. In cells and tissues coexpressing both IR and IGF-I-R, Hybrid-Rs, formed by one IR α and one β-subunit hemicomplex and one IGF-I-R α and β-subunit hemicomplex, have been observed (12–14). Functional studies with purified Hybrid-Rs indicate that they behave like IGF-I-Rs rather than IRs because they bind IGF-I with an affinity similar to that of the IGF-I-R, whereas they bind insulin with a much lower affinity (14, 15).

Our understanding of the biological role of Hybrid-Rs, when compared to IRs and IGF-I-Rs, has been hampered in part...
by the difficulty of directly measuring the absolute content of these Hybrid-Rs. Currently, Hybrid-Rs have been measured as the proportion of 125I-IGF-I binding immunoprecipitated by an anti-IR antibody (16). Thus, the presence, relative abundance, and functional role of Hybrid-Rs in human breast cancer are unknown.

In this report, we describe novel ELISAs that allow the direct measurement of the content of IRs, IGF-I-Rs, and Hybrid-Rs in breast cancer cells and specimens. These studies indicate that, in many breast cancers, the Hybrid-R is the major receptor that mediating the mitogenic response to IGF-I.

MATERIALS AND METHODS

The following materials were purchased as follows: MCD8-170 medium, FCS, bovine pituitary extract, and gentamicin obtained from Life Technologies, Inc. (Paisley, United Kingdom); and MEM, DMEM Nutrient Mixture-F-12 Ham’s medium (1:1 DMEM/F-12), and all chemicals, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). Tyr-A14-125I-labeled insulin (specific activity, 13.3 MBq/μg) was provided by Dr. R. Navalesi (Dipartimento di Endocrinologia e Metabolismo, University of Pisa, Pisa, Italy). 125I-labeled IGF-I (specific activity, 11.8 MBq/μg) was obtained from DuPont-NEN (Boston, MA). Antibodies to the IR and IGF-I-R, and Hybrid-Rs have been measured as by the difficulty of directly measuring the absolute content of these Hybrid-Rs. Currently, Hybrid-Rs have been measured as the proportion of 125I-IGF-I binding immunoprecipitated by an anti-IR antibody (16). Thus, the presence, relative abundance, and functional role of Hybrid-Rs in human breast cancer are unknown.

In this report, we describe novel ELISAs that allow the direct measurement of the content of IRs, IGF-I-Rs, and Hybrid-Rs in breast cancer cells and specimens. These studies indicate that, in many breast cancers, the Hybrid-R is the major receptor that mediating the mitogenic response to IGF-I.
chromatography on WGA-agarose and on agarose coupled either with the monoclonal antibody MA-20 (for IRs) or antibody αIR-3 (for IGF-I-Rs; Ref. 27). The Hybrid-R standard was obtained, as described previously (14), from human placental extracts by first immunocapturing Hybrid-Rs and IGF-I-Rs by a chromatographic step in αIR-3 antibody coupled agarose and then purifying Hybrid-R with a column containing agarose coupled to MA-51 antibody. This material was demonstrated to contain Hybrid-Rs by microsequencing, silver staining, and immunoblot (14). Receptor concentration was measured by amino acid analysis.

IR ELISA. IRs were captured by incubating cell or tissue lysates (0.5–60 μg/well) in Maxisorp immunoplates (Nunc, Roskilde, Denmark) precoated with 2 μg/ml anti-IR MA-20 antibody. After washing, the immunocaptured receptors were incubated with the biotinylated anti-IR CT-1 antibody [0.3 μg/ml in 50 mM HEPES-buffered saline (pH 7.6) containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF] and then with peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by the addition of 100 μl of 3.3’5,5’-tetramethylbenzidine [0.4 mg/ml in 0.1 M citrate-phosphate buffer (pH 5.0) with 0.4 μl/ml 30% H₂O₂]. The reaction was stopped by the addition of 1.0 mM H₂PO₄, and the absorbance was measured at 451 nm.

IGF-I-R ELISA. IGF-I-Rs were measured by immunocapturing receptors with anti-IGF-I-R αIR-3 antibody and using biotinylated anti-IGF-I-R 17-69 as a revealing second antibody. In both the IR and the IGF-I-R ELISAs, Hybrid-Rs (up to 1 ng/well) do not cause any appreciable interference.

Hybrid-R ELISA. Hybrid-Rs were measured by immuno
capturing receptors with anti-IR 83-7 antibody and using biotinylated anti-IGF-I-R 17-69 as a revealing second antibody.

Coprecipitation of Receptor-125I-Hormone Complexes. Assays were performed as described previously (16). Solubilized cells or tissues (20–40 μg of protein) were preincubated at 4°C with the radioactive ligand (20,000 cpm/50 μl for 125I-insulin or 10,000 cpm/50 μl for 125I-IGF-I; ~50–100 pm) in a final volume of 0.4 ml of 50 mM Tris-base buffer (pH 7.8) containing 200 kallikrein inhibitor units (KIU)/ml aprotinin, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF] and then with peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by the addition of 100 μl of 3.3’5,5’-tetramethylbenzidine, and the absorbance was measured at 451 nm.

IGF-I-R ELISA. IGF-I-Rs were measured by immunocapturing receptors with anti-IGF-I-R αIR-3 antibody and using biotinylated anti-IGF-I-R 17-69 as a revealing second antibody.

Rceptor Autophosphorylation ELISA. IR, IGF-I-R, and Hybrid-R autophosphorylation was measured by ELISA. A procedure similar to that described for receptor measurement was used. Cells in monolayer cultures were stimulated with increasing doses (0–100 nM) or either insulin or IGF-I for 5 min at 37°C. Cells were then solubilized, and receptors were captured by incubation of cell lysates in Maxisorp plates precoated with various antireceptor antibodies (MA-20, αIR-3, 17-69, and 83-7). After washing, a biotinylated antiphosphotyrosine antibody [4G10; Upstate Biotechnology, Inc.; 0.3 μg/ml in 50 mM HEPES-buffered saline (pH 7.6), containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF] was added to reveal phosphorylated receptors by the peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by the addition of 100 μl of 3.3’5,5’-tetramethylbenzidine, and the absorbance was measured at 451 nm, as described above.

Western Blot Analysis. To evaluate Hybrid-R activation by either insulin or IGF-I, we carried out Western blot analysis in subconfluent cell monolayers serum starved for 24 h in 0.1% BSA-MEM and incubated in the presence or absence 10 nM insulin or IGF-I and 50 nM receptor subtypes blocking antibodies (αIR-3 for IGF-I-R; 47-9 for Hybrid-Rs) for 5 min at 37°C. Cells were then solubilized with 50 mM HEPES (pH 7.4), 1% Triton X-100, and 2 mM PMSF. Solubilized extracts were first IR immunodepleted by incubation with protein A-Sepharose coated with monoclonal antibody MA-20 and subsequently immunodepleted of typical IGF-I-Rs by incubation with Protein A-Sepharose coated with antibody αIR-3. The remaining Hybrid-Rs were immunoprecipitated with antibody 83-7. After centrifugation at 10,000 × g for 5 min, the pellets were washed three times at high stringency [0.5 mM NaCl, 10 mM sodium phosphate (pH 7.4), 0.5% NP40, 2 mM EDTA, and 0.04% BSA], boiled in Laemmli buffer, and centrifuged at 10,000 × g for 5 min, and the supernatant was subjected to PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes that were then sequentially incubated with 1 μg/ml αPY and with a rabbit antimouse antiserum conjugated with horseradish peroxidase. The reaction was developed according to an enhanced chemiluminescence detection system (Amersham International, Amersham Place, United Kingdom).

To compare the autophosphorylation induced by IGF-I in typical IGF-I-Rs versus Hybrid-Rs, we exposed intact cell monolayers (MDA-MB157 or MCF-7) to IGF-I (10 nM). Cells were then solubilized, and receptors that were immunoprecipi-
tated with either αIR-3 or 83-7 antibodies were subjected to SDS-PAGE and immunoblotting with αPY, as described above.

To evaluate whether blocking antibodies to Hybrid-Rs inhibited IGF-I-R activation, we exposed MDA-MB157 or MCF-7 cell monolayers for 5 min to IGF-I (10 nM), together with either αIR-3 (antibody to IGF-I-R) or 47-9 (antibody to Hybrid-Rs). IGF-I-R and Hybrid-R autophosphorylation was then revealed by Western blot or by ELISA after immunoprecipitating or immunocapturing each receptor subtype with specific antibodies (αIR-3 and 83-7 for IGF-I-R and Hybrid-R, respectively).

Cell Growth Studies

To evaluate to what extent the mitogenic effect of IGF-I on breast cancer cells occurred via the IGF-I-Rs or the Hybrid-Rs, we measured the growth of MCF-7 or MDA-MB157 cells in the presence or the absence of various antireceptor blocking antibody (αIR-3 for IGF-I-R; 47-9 for Hybrid-Rs; MA-51 for IRs). Cells (5×10^3) were seeded in 96-well plates; after 24 h, the medium was removed and replaced with medium containing 2% charcoal-stripped FCS. After additional 24 h various concentrations of IGF-I (0–10 nM) were added in fresh medium with or without blocking antibodies. Cell growth was measured after 4 days, by measuring the rate of tetrazolium salts reduction to formazan, which is proportional to the number of living cells (EZ4U method; Biomedical, Wien, Austria). At the end of incubation, the absorbance was read at 450 nm.

In parallel experiments, cells were seeded in 25-mm multiwell plates at a density of 3×10^4 cells/well and incubated with IGF-I (with or without addition of antibodies) for 4 days with a medium change on day 3. Cells were detached with a 0.2% EDTA solution and counted in a hemochromocytometer. The cellular suspension was then centrifuged, the pellet was solubilized with 0.03% SDS, and the cellular DNA content was determined by a fluorimetric method.

Statistical Analysis

IR, IGF-I-R, and Hybrid-R cell and tissue contents were correlated by the Spearman rank correlation or by linear regression analysis after data transformation into natural logarithmics. Correlation of Hybrid-Rs with other variables was carried out by logistic regression. The statistical package SPSS (SPSS Inc., Chicago, IL) was used.

RESULTS

Measurements of Hybrid-Rs, IR, and IGF-I-R

Specific ELISAs. A variety of monoclonal antibodies to the IR and the IGF-I-R were screened for use in the Hybrid-R ELISA (Table 1). A sensitive and specific assay was developed by first capturing the Hybrid-R with IR α-subunit antibody 83-7 and reading out with biotinylated IGF-I-R α-subunit antibody 17-69 (Fig. 1A). The minimal detectable content of Hybrid-Rs was 0.125 ng/well (1.25 ng/ml). Neither purified IR nor purified...
IGF-I-R reacted in the assay (Fig. 1A). Moreover, there was no interference from the ligands, insulin, or IGF-I (data not shown). Multiple dilutions of extract of MDA-MB157 breast cancer cells and placenta tissue produced dose-response curves that paralleled those obtained with purified Hybrid-R standard (Fig. 2). Intra-assay CVs were <7% at 0.5 ng/tube and <8% at 1.0 ng/tube. Inter-assay CVs were <8% and <10%, respectively.

The ELISAs used for IR and IGF-I-R had similar features of sensitivity and specificity (Fig. 1, B and C, respectively). The minimal detectable content of IR was 0.05 ng/tube, and the minimal detectable content of IGF-I-R was 0.0625 ng/tube. For both assays, the intra-assay CVs were <8%, and interassay CVs were <10%.

**Comparison with the Coprecipitation Assay.** To compare the results obtained by this new Hybrid-R ELISA with the older coprecipitation method for Hybrid-R (16), we compared eight breast cancer cell lines and two nonmalignantly transformed cell line. With the coprecipitation method, the Hybrid-R content was evaluated as the proportion of total 125I-IGF-I binding that was precipitated by an anti-IR antibody.

The ELISA results obtained with purified Hybrid-R standard (Fig. 2) were repeats of those obtained with cultured cells (Table 2). Moreover, the ELISA results obtained with purified Hybrid-R standard (Fig. 2) were similar to those obtained with cultured cells (Table 2). The ELISA results obtained with purified Hybrid-R standard (Fig. 2) were similar to those obtained with cultured cells (Table 2). The ELISA results obtained with purified Hybrid-R standard (Fig. 2) were similar to those obtained with cultured cells (Table 2).

**Effect of Variation of IR Content on Hybrid-R Content**

To evaluate how changes of cell IR content may affect Hybrid-R content, we investigated the U937 human myeloid cell line. These cells have a relatively high IR content, which decreases by ~90% (from 5.3 ng/100 μg protein to 0.6 ng) when they differentiate into macrophages after treatment with 12-O-tetradecanoylphorbol-13-acetate. After 12-O-tetradecanoylphorbol-13-acetate treatment, the Hybrid-R content decreased from 3.8 to 1.5 ng, a value in close accordance with the values measured in cultured breast cells by specific ELISAs.

The value for Hybrid-R content predicted on the basis of random assembly of cell IR and IGF-I-R half-receptors correlated very closely with the measured cellular content of Hybrid-R (r = 0.810, P = 0.008). IR, IGF-I-R, and Hybrid-R were also measured in six breast cancer cell lines by Western blot analysis; receptor contents were very close to those found by ELISA (Fig. 3).

**Table 3**  IR, IGF-I-R, and Hybrid-R content, as measured in cultured breast cells by specific ELISAs

Predicted Hybrid-R content is calculated on the assumption of random assembly on the basis of IR and IGF-I-R cell content.

<table>
<thead>
<tr>
<th>Breast cells</th>
<th>Measured IR</th>
<th>Measured IGF-I-R</th>
<th>Measured Hybrid-R</th>
<th>Predicted Hybrid-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmalignant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-10</td>
<td>0.7</td>
<td>2.2</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>184B5</td>
<td>1.1</td>
<td>1.2</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Malignant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T47-D</td>
<td>0.4</td>
<td>10.0</td>
<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td>BT-20</td>
<td>0.5</td>
<td>7.6</td>
<td>3.0</td>
<td>3.9</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.0</td>
<td>34.0</td>
<td>10.0</td>
<td>11.6</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>2.6</td>
<td>2.6</td>
<td>6.0</td>
<td>5.1</td>
</tr>
<tr>
<td>ZR-75</td>
<td>5.0</td>
<td>10.0</td>
<td>9.0</td>
<td>14.1</td>
</tr>
<tr>
<td>MDA-MB157</td>
<td>11.0</td>
<td>2.0</td>
<td>12.0</td>
<td>9.2</td>
</tr>
<tr>
<td>MDA-MB468</td>
<td>15.0</td>
<td>1.2</td>
<td>16.0</td>
<td>8.6</td>
</tr>
<tr>
<td>MDA-MB453</td>
<td>16.0</td>
<td>1.8</td>
<td>17.8</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*If concentration of IR monomer is I, and that of IGF-I-R monomer is G, then, for random assembly, expected ratios are: IR(I):IGF-I-R (G):Hybrid-Rs (IG) = I^2:G^2:2IG. Therefore, predicted concentrations of Hybrid-Rs = \(\sqrt{IR \cdot IGF-I-R}\).*

**Table 2**  Proportion of Hybrid-Rs, as measured by both ELISA and coprecipitation assay methods

<table>
<thead>
<tr>
<th>Breast cells</th>
<th>ELISA (^a)</th>
<th>Coprecipitation (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmalignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-10</td>
<td>56.3</td>
<td>47.8</td>
</tr>
<tr>
<td>184B5</td>
<td>32.3</td>
<td>28.5</td>
</tr>
<tr>
<td>Malignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T47-D</td>
<td>27.5</td>
<td>36.4</td>
</tr>
<tr>
<td>BT-20</td>
<td>28.3</td>
<td>34.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>22.7</td>
<td>12.7</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>69.8</td>
<td>55.6</td>
</tr>
<tr>
<td>ZR-75</td>
<td>47.4</td>
<td>60.2</td>
</tr>
<tr>
<td>MDA-MB157</td>
<td>85.7</td>
<td>87.2</td>
</tr>
<tr>
<td>MDA-MB468</td>
<td>89.3</td>
<td>96.3</td>
</tr>
<tr>
<td>MDA-MB453</td>
<td>88.6</td>
<td>78.7</td>
</tr>
</tbody>
</table>

*Hybrid-R/(IGF-I-R + Hybrid-R) \times 100.

\(^b\) % of total 125I-IGF-I binding that was precipitated by an anti-IR antibody.
predicted by the random assembly model. IR overexpression, therefore, contributes to an increased Hybrid-R formation and to the signaling and biological effects of IGF-I.

**IR, IGF-I-R, and Hybrid-R Content in Human Breast Cancer Tissue Specimens**

The content of IRs, IGF-I-Rs, and Hybrid-Rs were then measured by ELISA in 39 breast cancer specimens, 9 breast fibroadenomas, and 18 normal breast specimens (Table 4). As reported previously (1, 2), the average content of both IR and IGF-I-R was significantly higher ($P < 0.0001$) in cancer tissues than in normal tissues and fibroadenoma tissue (Table 4). In addition, fat-free normal breast tissue was obtained by preparing organoids from three normal breast specimens obtained by reductive mammoplasty (26). Receptor content in normal breast tissue was very similar when measured either in fat-free or-reductive mammoplasty (26). Receptor content in normal breast tissue was not due to the contribution of adipose tissue (Table 4).

In 39 breast cancer specimens, the Hybrid-R content was $\sim 3$-fold greater than that of either IR or IGF-I-R. Hybrid-R content exceeded the IGF-I-R content in 30 cases. In all 39 breast cancer specimens, Hybrid-R content was significantly related to both IR ($r = 0.618, P = 0.0001$) and IGF-I-R content ($r = 0.576, P = 0.0001$, Spearman rank correlation), whereas the contents of IR and IGF-I-R were not correlated. Receptor content in benign breast tumors was intermediate between normal and cancer tissue but closer to that found in normal breast.

No significant association was found between cancer Hybrid-R content and the following variables: tumor estrogen or progesterone receptor content, tumor-node-metastasis stage, patient age or menopausal state, or presence of periluminal lymphocytic infiltration. Also in tissue specimens, the measured Hybrid-R values significantly correlated with values predicted on the basis of the random assembly model ($r = 0.735, P = 0.0001$). Hybrid-R content was correlated with both IR ($r = 0.576, P = 0.0001$) and IGF-I-R content ($r = 0.618, P = 0.0001$) in cancer tissues.

**Table 4** IR, IGF-I-R, and IR/IGF-I-R Hybrid content (ng/100 μg protein) in breast cancer specimens, breast fibroadenomas, and normal breast specimens

<table>
<thead>
<tr>
<th></th>
<th>IR</th>
<th>IGF-I-R</th>
<th>IR/IGF-I-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer tissue (n = 39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.50 ± 5.37</td>
<td>3.67 ± 6.57</td>
<td>6.04 ± 4.67</td>
</tr>
<tr>
<td>Range</td>
<td>0.2–25.0</td>
<td>0.3–39.5</td>
<td>0.8–28.0</td>
</tr>
<tr>
<td>Median</td>
<td>2.20</td>
<td>1.90</td>
<td>6.01</td>
</tr>
<tr>
<td>Fibroadenomas (n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.23 ± 1.64</td>
<td>0.74 ± 0.35</td>
<td>2.33 ± 1.92</td>
</tr>
<tr>
<td>Range</td>
<td>0.4–5.0</td>
<td>0.2–1.3</td>
<td>1.0–6.9</td>
</tr>
<tr>
<td>Median</td>
<td>1.55</td>
<td>0.78</td>
<td>1.60</td>
</tr>
<tr>
<td>Normal breast (n = 18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal breast specimens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.0 ± 1.1</td>
<td>0.50 ± 0.31</td>
<td>1.12 ± 0.87</td>
</tr>
<tr>
<td>Range</td>
<td>0.1–4.8</td>
<td>0.1–1.2</td>
<td>0.1–2.8</td>
</tr>
<tr>
<td>Median</td>
<td>0.65</td>
<td>0.41</td>
<td>0.77</td>
</tr>
<tr>
<td>Organoids (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.94 ± 0.04</td>
<td>0.84 ± 0.28</td>
<td>0.88 ± 0.26</td>
</tr>
<tr>
<td>Range</td>
<td>0.89–0.98</td>
<td>0.55–1.1</td>
<td>0.6–1.1</td>
</tr>
<tr>
<td>Median</td>
<td>0.95</td>
<td>0.87</td>
<td>0.95</td>
</tr>
</tbody>
</table>

0.69, $P = 0.0001$) and IGF-I-R content ($r = 0.75, P = 0.0001$) also in normal breast and in fibroadenomas.

**Hybrid-R, IGF-I-R, and IR Autophosphorylation in Breast Cancer Cells in Response to IGF-I or Insulin (by ELISA and Western Blot)**

We then studied Hybrid-R tyrosine kinase activity in breast cancer cells after stimulation by either insulin or IGF-I or both. MDA-MB157 cells were stimulated with either insulin or IGF-I and lysed, and receptor autophosphorylation was evaluated by ELISA after capture with four specific monoclonal antibodies (Fig. 4). When IGF-I-Rs were immunocaptured with the monoclonal antibody αIR-3, IGF-I but not insulin induced a dose-dependent receptor autophosphorylation ($EC_{50} = 0.2 $ nM; Fig. 4A). In contrast, when IR were captured with monoclonal antibody MA-20, an autophosphorylation occurred in response to insulin (EC$_{50}$ = 0.5 nM; Fig. 4B) but not in response to IGF-I. Hybrid-Rs were immunocaptured with two different antibodies (the anti-IR antibody 83-7 and the anti-IGF-I-R antibody 17-69). As expected, IGF-I stimulated autophosphorylation in both conditions (EC$_{50}$ = 0.35–0.5 nM; Fig. 4, C and D), whereas insulin stimulated autophosphorylation only when IRs were present (immunocapturing antibody, 83-7; Fig. 4C).

Western blot analysis of Hybrid-R autophosphorylation was also carried out in MDA-MB157 cells stimulated with either IGF-I or insulin. Cell extracts were first immunodepleted of both typical IRs and IGF-I-Rs using antibodies MA-20 and αIR-3, and then Hybrid-Rs were immunoprecipitated with antibody 83-7. As expected, Hybrid-Rs were autophosphorylated in response to IGF-I but not to insulin (data not shown). These data indicate that, as shown previously for isolated receptors (15), in intact breast cancer cells, Hybrid-Rs function as IGF-I-Rs.

**Biological Effect of Hybrid-R Stimulation by IGF-I**

Receptor Autophosphorylation and Blockade by Monoclonal Antibodies. To evaluate the significance of Hybrid-R in mediating the biological effects of IGF-I, we studied both
MCF-7 and MDA-MB157 breast cancer cells, each of which has a different IGF-I-R:Hybrid-R ratio. Cells were first exposed to IGF-I (10 nM), and either the IRs, IGF-I-Rs, or Hybrid-Rs were immunoprecipitated, subjected to SDS-PAGE, and blotted with anti-PY antibody (Fig. 5). IGF-I-stimulated autophosphorylation of IGF-I-Rs was high in MCF-7 cells but relatively low in MDA-MB157 cells. In contrast, IGF-I stimulated Hybrid-R autophosphorylation was relatively low in MCF-7 cells but relatively high in MDA-MB157 cells. These data were in close accordance with the different IGF-I-R:Hybrid-R ratios in these cell lines (Table 2).

Receptor autophosphorylation was then measured in cells exposed to 10 nM IGF-I in the presence or absence of blocking antibodies that were specific for either the IGF-I-R (αIR-3) or Hybrid-R (Ab 47-9). As expected, blockade with αIR-3 markedly inhibited IGF-I-R activation but not Hybrid-R activation. In contrast, Ab 47-9 inhibited Hybrid-R activation (Fig. 6, A and B). The latter effect was more evident in MDA-MB157 cells, in which Hybrid-Rs represented the majority of IGF-I binding sites.

Mitogenesis after Blockade by Monoclonal Antibodies.

We then evaluated the effect of receptor blockade on cell growth. IGF-I stimulation of growth was most strongly inhibited by Ab 47-9 (Hybrid-R blocking antibody) in MDA-MB157 cells, in which Hybrid-Rs represent the majority of IGF-I binding sites (Fig. 7A). In contrast, IGF-I stimulation of growth was most strongly inhibited by αIR-3 (IGF-I-R blocking antibody) in MCF-7 cells, in which IGF-I-Rs represent the majority of IGF-I binding sites (Fig. 7B). As expected, blockade of IRs by antibody MA-51 did not affect IGF-I-stimulated growth in either cell type.

DISCUSSION

We previously reported that the IR and the related receptor IGF-I-R are overexpressed in breast cancers (1, 3) and that both the IR and IGF-I-R are expressed at high levels in 45% of these cancers. Interestingly, IR content exceeds IGF-I-R content in ~40% of cases, whereas IGF-I-R content exceeds IR content in 15% of cases (7). Using immunohistochemistry, we observed...
that IRs were overexpressed by the neoplastic epithelial cells and that the content of IR in these cells was related to clinical outcome (1, 9). IR expression was a major predictor of reduced disease-free survival in lymph node-negative breast cancers (9). Because insulin is not locally produced in breast cancer tissues, it was not known by what mechanism the IR was activated, thus leading to altered cellular behavior.

There are several lines of evidence that the growth factor IGF-I plays a major role in breast cancer. (a) It is a potent transforming and mitogenic agent (11). (b) It is locally produced by breast cancer stromal cells (10). (c) Its receptor, the IGF-I-R, is overexpressed in most breast cancer cells. (d) IGF-I-R expression is an obligatory requirement both for developing a transformed phenotype and protecting cancer cells from apoptosis (28). For these reasons, the IGF-I/IGF-I-R ligand-receptor paracrine loop has been the target of a variety of strategies aiming to inhibit IGF-I signaling in cancer cells (29–31). In contrast, the IR binds IGF-I with low affinity and, thus, does not participate in this loop.

In addition to homotypic IRs and IGF-I-Rs formed by identical αβ half-receptors that are disulfide-linked into ααββ tetramers, the existence of heterotypic Hybrid-Rs has been shown in a variety of human tissues and cultured cells expressing both IR and IGF-I-R (32). Hybrid-Rs are heterotetrameric complexes that are formed because a proportion of IR αβ half-receptors assemble with IGF-I-R αβ half-receptors, given the high degree of homology between these two receptors. Assembly of αβ half-receptors into either homotypic IRs and IGF-I-Rs or Hybrid-Rs appear to result from a random process (32): a high proportion of Hybrid-Rs results, therefore, from the expression of a large excess of one of the two homotypic receptors (33).

Purified Hybrid-Rs bind ~20 times as much IGF-I than insulin, and IGF-I has a greater ability than insulin to stimulate Hybrid-R autophosphorylation, which occurs by intramolecular trans-phosphorylation of both β-subunits (33, 34). These studies suggest, therefore, that Hybrid-Rs behave like IGF-I-Rs, although their functional importance in both normal and neoplastic tissues is not known.

Herein, we document that IR overexpression in breast cancer that IRs were overexpressed by the neoplastic epithelial cells and that the content of IR in these cells was related to clinical outcome (1, 9). IR expression was a major predictor of reduced disease-free survival in lymph node-negative breast cancers (9). Because insulin is not locally produced in breast cancer tissues, it was not known by what mechanism the IR was activated, thus leading to altered cellular behavior.

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Finally, we studied the effect of monoclonal antibodies specific to either Hybrid-R or IGF-I-R on the mitogenic effect of IGF-I. Cell growth studies in the presence of these antibodies indicated that, in a given cell type, the IGF-I mitogenic effect occurred predominantly via the receptor type that was more abundant. Thus, an anti-IR antibody that recognized the Hybrid-R (Ab 47-9) substantially blocked growth in breast cancer cells that had a high Hybrid-R:IGF-I-R ratio. In contrast, an anti-IGF-I-R antibody that recognizes the IGF-I-R (αIR-3) substantially blocked growth in breast cancer cells that had a high IGF-I-R:Hybrid-R ratio.

In summary, these data provide new insights into the role of IR overexpression in breast cancer by demonstrating that IR overexpression increases the cellular response to IGF-I by increasing Hybrid-R formation. Moreover, in light of these data, a reconsideration of therapeutic strategies for breast cancer based on the blockade of the IGF-I/IGF-I-R loop may be needed. A more effective receptor-based strategy may occur when both the IGF-I-R and the IR are chosen as targets for down-regulation. Furthermore, a more effective blockade of IGF-I signaling may be obtained by using either antibodies or other agents that block IGF-I binding to both Hybrid-Rs and the IGF-I-Rs.

REFERENCES


Insulin and Insulin-like Growth Factor-I (IGF-I) Receptor Overexpression in Breast Cancers Leads to Insulin/IGF-I Hybrid Receptor Overexpression: Evidence for a Second Mechanism of IGF-I Signaling

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