Elevated Insulin-like Growth Factor I Receptor Autophosphorylation and Kinase Activity in Human Breast Cancer

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ABSTRACT

Insulin-like growth factor I action has been implicated in the pathogenesis of many different malignancies, including breast cancer. Insulin-like growth factor I receptors (IGF-IRs) are overexpressed in virtually all breast cancer cell lines, in which they are believed to enhance growth and inhibit apoptosis. In this study, the functional activity of IGF-IRs from normal and malignant human breast tissue was assessed. IGF-IR expression was 14-fold higher in malignant breast tissue than in normal breast tissue. IGF-IR autophosphorylation and kinase activity were 2-4-fold higher in purified receptor preparations from malignant breast tissue compared to normal breast tissue when normalized for receptor number. This increase in receptor function, coupled with the enhanced receptor expression, amounts to a 40-fold elevation in IGF-IR tyrosine kinase activity in malignant breast tissue. The enhanced receptor autophosphorylation and kinase activity were observed in the absence of hormonal stimulation and seem to result from an alteration in the intrinsic activity of the receptor itself. Protein tyrosine phosphatase activity is also increased in malignant breast tissue. These data suggest that the IGF-IR is an important target for breast cancer therapy.

INTRODUCTION

The IGF-IR is necessary for normal progression through the cell cycle and for normal growth and development. Embryonic fibroblasts derived from IGF-IR knockout mice demonstrate prolongation of all phases of the cell cycle compared to cells from normal littermates. Mouse embryos without IGF-IRs were not viable at birth due to defects of the lung, nervous system, skin, and bones (1-3). The IGF-IR, however, causes abnormal growth regulation when overexpressed (4). There is a substantial amount of evidence suggesting that defects of the lung, nervous system, skin, and bones (1-3). The IGF-IR plays a prominent role in cellular transformation, mitosis, and, importantly, breast cancer (11-15).

MATERIALS AND METHODS

Receptor Purification Protocol. Normal and malignant human breast tissue specimens were obtained as soon as possible (<8 h) after resection, frozen immediately in liquid nitrogen, and stored at ~70°C until use. Frozen specimens were solubilized with a Polytron homogenizer in 1 ml of solubilizing fluid per 100 mg of tissue. The solubilization buffer was EBG [120 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 25 mM HEPES (pH 7.4), and 10% glycerol] containing 1% Triton X-100 with multiple protease inhibitors (2.5 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml bacitracin, 800 KIU/ml aprotinin, and 15 mM benzamidine). The sample was placed on ice for 10 min and then centrifuged for 30 min at 800 × g at 4°C. The fat cake was removed, and the infranatant was collected and clarified by centrifugation for 25 min at 100,000 × g at 4°C. The second supernatant was applied to a WGA (Vector Laboratories, Inc., Burlingame, CA) affinity chromatography column (0.5 ml/gm tissue) to collect glycosylated proteins at 4°C. The column was washed with 60 ml of cold EBG, and the proteins were eluted with elution buffer (0.3 M N-acetyl-glucosamine in EBG). The WGA-purified receptors were stored at ~70°C. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA).

Binding Studies on WGA-purified Receptors from Human Breast Tissue. Binding studies were performed to determine the level of expression of the IGF-I and insulin receptors in the normal and malignant tissues. Aliquots (20 μl) of the WGA-purified receptors were incubated with 0.13 nM 125I-labeled IGF-I or insulin for 16 h at 4°C in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100. BSA (0.1%) was added to the insulin binding reactions to block nonspecific binding but was omitted from the IGF-I binding reactions, because it was found to contain IGF-I activity. Binding competition curves were generated using increasing concentrations of unlabeled insulin, IGF-I, or des (1-3) IGF-I (Gro-Pep, Adelaide, Australia), an IGF analogue that binds with normal affinity to the IGF-IR but does not bind to IGF-binding proteins. Free and bound ligand were separated by polyethylene glycol precipitation. Binding data were analyzed by Scatchard analysis to determine receptor number. Initial experiments showed that the affinity of the receptor for IGF-I was similar in malignant and normal tissue. Because of limited amounts of malignant tissue, subsequent experiments only used cold ligand at 6.6 and 263 nM to determine receptor number.

In Vitro Autophosphorylation and Kinase Assay. WGA-purified receptors were stimulated and allowed to phosphorylate in the presence of ATP to compare the activity of receptors from normal and malignant tissue. WGA-purified receptors (50-100 fmol) were diluted with elution buffer (0.3 M N-acetyl-glucosamine in EBG) with 2 mM orthovanadate. The receptors were stimulated with IGF-I, IGF-II, or insulin at either 0.1, 3, or 10 nM for 30 min at 4°C. A sample of purified IGF-IRs from a CHO cell line engineered to
overexpress the IGF-IR was used as an internal control in each experiment. Phosphorylation was initiated by adding 10 μl of a kinase buffer composed of 25 μM ATP, 5 mM MnCl₂, 20 mM MgCl₂, 10 mM HEPES (pH 7.4), and 0.05% Triton. This buffer was added along with 2 μg of IRS-GST, a recombinant protein that serves as a substrate for IGF-IR and insulin receptor kinase activity. This substrate, consisting of the last 300 aa of IRS-1 (aa 1132-1432) was expressed as pmol of 32P phosphate released/min/mg protein. The reaction was terminated by adding 150 μl of ice-cold 10% trichloroacetic acid, and samples were centrifuged at 14,000 × g rpm at 4°C for 5 min. Supernatant (200 μl) was added, and the sample was vortexed again for exactly 10 s and then centrifuged for exactly 10 s. 160 μl of 5% ammonium-molybdate in 2 M H₂SO₄ were added, and the sample was vortexed again for exactly 10 s and then centrifuged for exactly 10 s. The pellets were washed four times with EBG plus 2 mM vanadate (EBGV), and resuspended in 27.5 μl of EBGV. The IGF-IR bound to the antibody complex was allowed to autophosphorylate in the absence of ATP. As can be seen, none of the samples demonstrated elevated basal autophosphorylation and kinase activity seen, immunoprecipitated with a-IR3, an IGF-IR-specific antibody, was precipitated with IGF-IR antibody, a-IR3, was then added to a final concentration of 15 μtg/ml and incubated for 4 h at 4°C. Receptor-antibody complexes were precipitated with 100 μl of protein G-Sepharose for 1 h at 4°C. The pellets were washed four times with EBG plus 2 mM vanadate (EBGV), and resuspended in 27.5 μl of EBGV. The IGF-IR bound to the antibody complex was allowed to phosphorylate by the addition of 10 μl of kinase buffer [25 μM ATP, 5 mM MnCl₂, 10 mM HEPES (pH 7.4), and 0.05% Triton X-100] and 1 μg of IRS-GST and incubated for 30 min at 25°C. The reaction was terminated by boiling with Laemmli’s sample buffer; the proteins were separated by SDS-PAGE on a 7.5% gel and then transferred to nitrocellulose membranes. Tyrosine-phosphorylated proteins were visualized by enhanced chemiluminescence (Amer sham). Data were quantified by densitometric scanning of the autoradiograms with the NIH Image Program (Version 1.6) on an Agfa Arcus II scanner.

**Immunoprecipitation of the IGF-IR.** Aliquots of WGA-purified receptors (100 fmol) were diluted to a volume of 50 μl with elution buffer (0.3 M N-acetyl-glucosamine in EBG) and 2 mM orthovanadate and then stimulated with 0.1, 1, or 3 μg of IGF-I. The samples were incubated at 30°C for 40 min. The IGF-IR antibody, a-IR3, was then added to a final concentration of 15 μg/ml and incubated for 4 h at 4°C. Receptor-antibody complexes were precipitated with 100 μl of protein G-Sepharose for 1 h at 4°C. The pellets were washed four times with EBG plus 2 mM vanadate (EBGV), and resuspended in 27.5 μl of EBGV. The IGF-IR bound to the antibody complex was allowed to phosphorylate by the addition of 10 μl of kinase buffer [25 μM ATP, 5 mM MnCl₂, 10 mM HEPES (pH 7.4), and 0.05% Triton X-100] and 1 μg of IRS-GST and incubated for 30 min at 25°C. The reaction was terminated by boiling with Laemmli’s sample buffer; the proteins were separated by SDS-PAGE on a 7.5% gel and then transferred to nitrocellulose membranes. Tyrosine-phosphorylated proteins were visualized as described above.

**PTPase Assay.** PTPase activity was measured using as substrate a synthetic insulin receptor peptide corresponding to the major sites of tyrosine autophosphorylation (aa 1142-1153). The peptide, containing three potential sites of tyrosine phosphorylation, was phosphorylated in vitro by purified human insulin receptors in the presence of [γ-32P]ATP. Briefly, the peptide (1 mg) was dissolved in 123 μl of 0.8% triethylamine in H₂O; neutralized with 0.5 μl of HOAc, 1.26 μl of HEPES (pH 7.4), and 1.26 μl of 10% Triton X-100; and added to the activated insulin receptors along with 50 μl of [γ-32P]ATP (10 μCi/μl, 3,000 Ci/mmol). After overnight incubation at 4°C, the reaction was terminated. The dephosphorylation reaction was carried out at 30°C in a final volume of 110 μl, with 1-5 μM 32P-labeled insulin receptor peptide (average of 50,000 cpm). After 9 min, the reaction was terminated by adding 150 μl of ice-cold 10% trichloroacetic acid, and samples were centrifuged at 14,000 × g rpm at 4°C for 5 min. Supernatant (200 μl) was removed and combined with 0.8 ml of ice-cold 5 mM silico-tungstic acid in 1 mM H₂SO₄ and 1.2 ml of isobutanol/toluene (1:1). The solution was vortexed for exactly 30 s, 160 μl of 5% ammonium-molybdate in 2 M H₂SO₄ were added, and the sample was vortexed again for exactly 10 s and then centrifuged at 1,000 × g rpm for 4 min at 4°C. Supernatant (800 μl) was then removed and counted to determine phosphate activity. The specific activity of the enzyme was expressed as pmol of 32P phosphate released/min/mg protein. The reaction was linear with respect to time and amount of PTPase activity present until at least 40% of the 32P phosphate had been released. All determinations were performed within the linear range, and all assays were done in duplicate.

**RESULTS**

**IGF-I and Insulin Receptor Expression Is Increased in Malignant Breast Tissue.** A total of 22 malignant and 20 normal human breast tissue samples were studied. IGF-I and insulin receptor levels were determined by performing binding competition experiments with 125I-labeled IGF-I and 125I-labeled insulin. Significant binding of 125I-labeled IGF-I to IGF-binding proteins was excluded by performing some experiments with an IGF-I analogue, des (1-3) IGF-I, which binds exclusively to the IGF-IR. Fig. 1A depicts full IGF-I competition curves generated for eight samples (five malignant and three normal). Scatchard analyses show a single IGF-I binding site (Fig. 1B) and significant variability in receptor number between normal and malignant samples. Because there were no significant differences in affinity among the eight samples, and because of the limited sample size of the malignant tissues, receptor levels in subsequent samples were determined using des (1-3) IGF-I competition at 6.6 and 263 nM. IGF-I and insulin binding are expressed as femtomoles bound/gram of tissue (Fig. 1C). The mean IGF-I and insulin binding are elevated 14-fold in the malignant specimens, in agreement with previous publications (16-20). IGF-IR expression is 3-4 times greater than insulin receptor expression.

**Receptor Autophosphorylation and Kinase Activity Are Elevated in Malignant Tissue.** The experiments in this section were designed to test whether or not the IGF-IRs in malignant breast tissue are functionally normal. Receptor autophosphorylation and kinase activity were assessed in 12 paired malignant and normal breast tissue samples. Equal amounts of receptor (50-100 fmol, based on the binding results) were allowed to autophosphorylate in the presence of a kinase substrate composed of the COOH-terminal domain of IRS-1 fused to GST. Tyrosine phosphorylation was visualized by antiphosphotyrosine immunoblotting and chemiluminescence and quantified by densitometric scanning of the autoradiographs. Representative autoradiographs from a malignant and a normal sample are seen in Fig. 2, A and B. This sample had few insulin receptors, and the last three lanes in the autoradiograph of the malignant tissue are overloaded to normalize for insulin receptor number. Each pair of experiments was normalized to the CHO control run on each gel, and the results are expressed relative to the basal activity of the normal sample.

The mean autophosphorylation activity is shown in Fig. 3A, and kinase activity is shown in Fig. 3B. Autophosphorylation and kinase data were analyzed by ANOVA. ANOVA indicated statistically significant differences between the columns (P < 0.0001) for both autophosphorylation and kinase activity. Autophosphorylation in response to IGF-I, IGF-II, and insulin is increased roughly 2-3-fold in malignant as compared to normal tissue, and kinase activity is increased 3-4-fold. Interestingly, this increase in protein tyrosine phosphorylation is seen in the absence of hormonal stimulation in the malignant samples, suggesting a mechanism of receptor activation independent of ligand. Linear regression was performed to determine whether the increase in kinase activity is correlated to the increase in autophosphorylation. This correlation was extremely significant with a P < 0.0001 and a correlation coefficient = 0.40 (Fig. 3C).

One facile explanation for the elevated basal tyrosine kinase activity is that the receptors are being purified in a preactivated and phosphorylated state from the malignant tissue. Five malignant and four normal samples were subjected to the autophosphorylation assay in the absence of ATP. As can be seen, none of the samples demonstrates any tyrosine-phosphorylated proteins (Fig. 4). Hence, phosphorylated receptors are not present in the WGA extracts.

To prove that the IGF-IR kinase was primarily responsible for the elevated basal autophosphorylation and kinase activity seen, immunoprecipitation with α-IR3, an IGF-IR-specific antibody, was performed (Fig. 5). The elevated basal autophosphorylation and kinase activity precipitated, indicating that the increase in basal autophosphorylation, as well as the ligand-stimulated activity, results from the IGF-IR itself rather than from other tyrosine kinases present in the WGA extract. We cannot, however, exclude the possibility that the...
increase in IGF-IR and substrate phosphorylation is the result of a tightly associated kinase that communoprecipitates with the IGF-IR.

PTPase Activity. Cellular tyrosine phosphorylation levels are maintained by a balance between kinase and phosphatase activity. An alteration in the phosphatase activity of the malignant samples could account for the observed increase in tyrosine phosphorylation. In an attempt to account for the differences in autophosphorylation and kinase activity in malignant and normal breast tissue, total phosphatase assay was assessed. Assays were performed on both total lysates (22 malignant and 20 normal samples) and with WGA extracts (9 malignant and 10 normal samples). Unexpectedly, the malignant samples had elevated phosphatase activity when normalized for protein in both the total lysates and the WGA-purified fraction. PTPase activity was found to be elevated 4-fold in malignant tissue as compared to normal tissue ($P < 0.0001$; Fig. 6).

**DISCUSSION**

We confirm in this study that IGF-IRs are overexpressed in WGA extracts of malignant breast tissue. More importantly, we show that basal autophosphorylation and kinase activity of equal femtomoles of IGF-IR were elevated 3–4-fold in malignant breast tissue, and that this increase was maintained on ligand stimulation. The increased tyrosine kinase activity in the extracts was highly correlated with autophosphorylation of the β subunit of the IGF-IR, suggesting that the kinase activity is from the receptor itself. This conclusion is further supported by communoprecipitation of the IGF-IR before measuring tyrosine kinase activity. The
overexpression of the receptors combined with the increased tyrosine kinase activity results in an increase in IGF-IR tyrosine kinase activity of ~40-fold in malignant tissue.

IGF-I and insulin receptor overexpression in breast malignancy has previously been reported. Using an IGF-IR RIA, Papa et al. (18) reported that the average IGF-IR content in 184 human breast cancers was 10-fold higher than the value obtained in normal breast tissue. They observed a positive correlation between IGF-IR content and insulin and estrogen receptor content but observed no correlation with tumor parameters including lymph node involvement, tumor size, or grade. Of interest, IGF-IR content was higher in a group of 129 low-risk specimens than in a group of 136 high-risk specimens, indicating that IGF-IR content is a favorable prognostic indicator (18). Other authors have reported similar results (16–19).

Conversely, a
Recent study has highlighted a link between IGF-IR overexpression and local breast cancer recurrence after lumpectomy and radiation therapy but not with overall survival (22). Insulin receptor content of human breast cancer specimens has previously been reported to be 6-fold higher than that of normal tissue (23). Insulin receptor and IGF-IR overexpression is potentially mediated by inactivation of p53 through derepression of the gene promoter (20, 24). The function of the IGF-IRs in primary human breast tissue has not previously been reported, although the overall protein tyrosine kinase activity is known to be elevated in breast malignancy as compared to normal breast tissue (25-28).

Our study focused on the autophosphorylation and tyrosine kinase activity of receptors partially purified by lectin chromatography. Both IGF-I and insulin receptors are present in these preparations. The most striking finding of these studies is the increase in phosphorylation and kinase activity of the receptors in the absence of ligand. We theorized that a loss of phosphatase activity could therefore result in tyrosine phosphorylation and a concomitant decrease in IRS-1-associated phosphatidylinositol 3'-kinase activity (46). Previous studies have suggested that IGF-IR content correlates with a better prognosis (18). This may be because IGF-IR content correlates with ER content, and ER-positive tumors are responsive to hormonal therapy.

The increase in IGF-IR autophosphorylation and kinase activity seems to represent a change in the intrinsic properties of the receptor. For example, a subtle structural alteration could occur during malignant transformation, resulting in constitutive receptor activation. All receptors are not fully activated, however, because hormonal stimulation causes a similar incremental increase in autophosphorylation and kinase activity of receptors from the malignant tissue. Because the basal kinase activity precipitates with the IGF-IR, we believe it results from the receptor itself. It is possible that a tightly associated kinase could coprecipitate; however, we did not observe any other tyrosine-phosphorylated proteins in the complex, arguing against the presence of a tyrosine kinase. Similarly, no additional proteins were detected using Ponceau S as a nonspecific protein stain.

Although the precise mechanism remains elusive, the observed overexpression of the IGF-IR, coupled with the significant increase in autophosphorylation and kinase activity in malignant breast tissue, has significant implications for breast cancer. These data show that the IGF-IR and its signaling pathway are important targets for breast cancer therapy. Much support for this conclusion already exists in the literature. Overexpression of the IGF-IR results in cell transformation (4), and transfection of a mutant dominant-negative IGF-IR reverses the transformed phenotype (9, 10). Studies on the mechanism by which the src oncogene transforms cells have identified the IGF-IR as a functionally significant pp60^c-src substrate (32, 33). Baserga and colleagues have elegantly demonstrated that the IGF-IR is important not only in cell transformation, but also in the inhibition of apoptosis (5-8). Furthermore, the overexpression of the IGF-IR decreases cellular sensitivity to radiation and chemotherapeutic agents (22, 34). Without the presence of a functional IGF-IR, oncogenes such as SV-40 T-antigen and the epidermal growth factor receptor are not transforming (5-8). In nude mice, mutant IGF-IRs or antisense to the IGF-IR mRNA prevents ligand-dependent cell transformation and induces apoptosis in a variety of human cancer cells implanted s.c. (9-15). IGF-1 action also plays a role in cellular migration in vitro and may play a role in the metastasis of breast cancer in vivo (35-38). Finally, mitogen-activated protein kinase, a downstream signaling molecule of the IGF-IR and many other tyrosine kinase growth factor receptors, is heavily tyrosine phosphorylated and is overexpressed 5-20-fold in human breast cancer specimens (39).

Increasing evidence suggests that the GH:IGF-1 axis is important in the pathogenesis of breast cancer. MCF-7 cells grew much less well in nude mice homozygous for the lit mutation, a missense mutation resulting in loss of function of the pituitary GH-releasing hormone receptor and secondary suppression of GH and IGF-I, than in control animals (40). Antiestrogen therapy for breast cancer may achieve some of its effect by modulating the GH:IGF-1 axis (41-45). For example, tamoxifen administration in humans causes a reduction in serum IGF-I concentrations by inhibiting both GH secretion and the local production of IGF-I in the lung and liver, common sites of breast cancer metastasis. Antiestrogens down-regulate IGF-IR expression, whereas IGF-I, IGF-II, or insulin in combination with estradiol are synergistic in their effects on the MCF-7 breast cancer cell line in culture. The cytostatic effect of tamoxifen, however, is not simply due to the repression of IGF-IR expression, because a similar effect is observed in MCF-7 cells engineered to overexpress the IGF-IR. In these cells, tamoxifen caused a decrease in IGF-IR and IRS-1 tyrosine phosphorylation and a concomitant decrease in IRS-1-associated phosphatidylinositol 3'-kinase activity (46). Previous studies have suggested that IGF-IR content correlates with a better prognosis (18). This may be because IGF-IR content correlates with ER content, and ER-positive tumors are responsive to hormonal therapy.
Our study further highlights the significance of IGF-IR signaling as a target for breast cancer therapy by demonstrating the novel finding of enhanced receptor activation in addition to receptor overexpression. Because this increased receptor function is ligand independent, therapeutics targeting IGF-IR activation or postreceptor signal transduction are likely to be more effective than those inhibiting IGF-IR binding.

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REFERENCES

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