

Insulin-like Growth Factor 1 Receptors in Human Breast Cancer and Their Relation to Estradiol and Progesterone Receptors¹

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ABSTRACT

Insulin-like growth factor 1 (IGF1) binding sites were characterized in breast cancer. We demonstrate the presence of one high affinity binding site. Chemical cross-linking of ¹²⁵I-IGF1 to breast cancer membranes in reducing condition and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed one band with an apparent molecular weight of 130,000. The specificity of the binding was studied. IGF2 was a good competitor whereas insulin competed with a potency lower than 1/100 that of IGF1. This IGF1 binding corresponded to the previously described type 1 IGF receptor (IGF1-R). IGF1-R was determined in 76 human breast cancer biopsies. Ninety-three % of the tumors were positive. The specific binding range was 0-16.4%; the geometric IGF1-R mean level was 3.9%. There was a relation (χ^2 test) between IGF1-R and progesterone receptor positivity rates ($P = 0.002$). The IGF1-R concentrations were correlated (Spearman test) with those of estradiol receptor ($P = 0.0018$) and progesterone receptor ($P = 0.0011$). A positive linear correlation existed between IGF1-R and estradiol receptor ($P = 0.006$) and between IGF1-R and progesterone receptor ($P = 0.003$). Our demonstration of the presence of IGF1-R in human breast cancer biopsies suggests that IGF1, acting either via the endocrine, paracrine, or autocrine pathways, could stimulate tumor growth.

INTRODUCTION

IGF1,² also termed somatomedin C, is a polypeptide the well-known physiological role of which is its contribution, with growth hormone, to the skeletal development via the endocrine pathway (1, 2). IGF1 is synthesized in many different tissues (3); recently it has been demonstrated that most if not all tissues express the IGF1 gene (4, 5); the finding of induction of IGF1 expression by estrogen in the uterus expands the role and significance of IGF1 as a mediator of growth beyond that related to GH (6). These results and the demonstration that IGF1 modulates the cycle of many cell types (7) suggest that this factor is very important in the growth of all tissue.

The involvement of IGF1 in the growth of human breast cancer cell lines has recently been demonstrated (8, 9). Moreover Baxter *et al.* (10) and Huff *et al.* (11) detected IGF1-secreted immunoreactivity in all the human breast cancer cells they studied. The use of complementary DNA probes to authentic IGF1 demonstrated that IGF1 is produced locally (11). The IGF1 production was not induced by estradiol in cells cultured with phenol red medium (11, 12) but subsequent studies utilizing phenol red-free medium showed a 2- to 3-fold increase in IGF1 production with estradiol or insulin treatment (13).

The first step of IGF1 action is its binding to membrane receptors. The receptor for IGF1 is remarkably similar to that of insulin, comprising a heterotetrameric structure with two α and two β subunits joined by disulfide bridges (14). IGF1-Rs

have been characterized by competitive binding and cross-linking techniques on cultured human breast cancer cell lines (8) and on breast cancer cell line membranes (15). IGF1-R presents, as in normal tissue, close homology with insulin receptor with a M_r 130,000 binding subunit. We have shown recently that the membranes extracted from breast cancer biopsies bind specifically IGF1 (15). These results indicate that IGF1 acting via the endocrine, paracrine, or autocrine pathways could be an important factor in the development of human breast cancer (16). It is therefore important for a better understanding of breast tumor biology to document the presence, absence, or alteration of IGF1-R.

In the present report IGF1-Rs in breast cancer are characterized and the level of these receptors in breast cancer biopsies is determined and related to the concentrations of estradiol and progesterone receptors in the same biopsies.

MATERIALS AND METHODS

Collection of Tumors. Tumor specimens, adenocarcinomas only, were obtained from 76 patients undergoing surgery for primary breast cancer in the Centre Oscar Lambret (Lille). At the time of collection, fat was removed and samples were divided into two parts; one was submitted for histological studies and the other was immediately frozen for receptor analysis. Our series was composed of 61 ductal, 9 lobular, 2 medullary, 1 ductal-lobular, 1 colloid, and 2 apocrine invasive carcinomas.

Tissue Processing. The frozen tissue was weighed and then pulverized (Spex-Bioblock, France). The tissues were homogenized in 20 mM Tris-3 mM EDTA-1 mM dithiothreitol-0.01% azide, pH 7.6.

The homogenate was centrifuged at $800 \times g$ for 10 min and the supernatant was ultracentrifuged at $105,000 \times g$ for 60 min. The supernatant (cytosol) was removed and the pellet ("microsomal" fraction) was resuspended in 25 mM Tris-HCl-10 mM MgCl₂-10⁻⁴ M phenylmethylsulfonyl fluoride buffer, pH 7.6. The protein concentration was determined by the method of Lowry *et al.* (17) applied either directly in the cytosol fraction or after extraction from the membranes (with 1 N NaOH) in the microsomal fraction.

IGF1 Labeling. The human native IGF1 and IGF2 were a generous gift from Dr. Humbel (Zurich, Switzerland). Human synthetic IGF1 was purchased from Amersham (ARN 4010; Amersham-France, Paris, France). A modification of the method of Hunter and Greenwood (18), using 800 ng chloramine-T and 1 μ g IGF1 with incubation for 50 s at 23°C, was used to iodinate IGF1. Iodinated IGF1 was purified on an ACA-54 column (LKB, France) and the tubes comprising the radioactive protein peak were diluted in assay buffer and could be stored as long as 2 weeks at 4°C. Specific activities, as calculated by isotope recovery, ranged between 160 and 220 μ Ci/ μ g. The quality of the preparation was checked after each iodination using a standard laboratory preparation of BT-20 breast cancer cell line membrane receptors. When 400 μ g of the usual protein membrane preparation were utilized, at least 10% of the iodinated IGF1 was specifically bound when the tracer was considered acceptable.

IGF1-R Assay. Unless otherwise specified, for the binding test 400 μ g of membrane proteins were incubated for 5 h at 4°C with approximately 200,000 cpm of iodinated IGF1 in the presence or absence of an excess of IGF1 crude preparation (the crude mixture of IGF1 and IGF2 was a generous gift from Dr. Humbel). The final incubation volume was adjusted to 0.5 ml with Tris-MgCl₂ buffer containing 0.1%

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² The abbreviations used are: IGF1, IGF2, insulin-like growth factors 1 and 2; IGF1-R, IGF1 receptor; ER, estradiol receptor; PgR, progesterone receptor.

bovine serum albumin (fraction V, Ref. A3912; Sigma Chemical Company, St. Louis, MO). The receptor assay was carried out at the same time; it was repeated once. Duplicates were used for total and nonspecific determination. In each series, a characterized pool of cell membrane receptors (BT-20) was included to ensure the assay quality control. A tumor was considered positive (IGF1-R positive) when the specific binding was higher than 1% (expressed as the percentage of the total radioactivity added). We used 1% for the positive threshold, since when the assay had been performed on boiled membranes, *i.e.*, on membranes with denaturated receptors, the difference between the binding in the absence or presence of an excess of IGF1 was always less than 1%.

Chemical Cross-Linking of ^{125}I -labeled IGF1. Membrane preparations (800 μg) were incubated with approximately 8×10^5 cpm ^{125}I IGF1 for 5 h at 4°C in the absence or presence of excess unlabeled IGF1. Membranes were then washed twice by the addition of 3 ml cold 10 mM sodium phosphate buffer, pH 7.4, and centrifuged at $3,000 \times g$ for 20 min. The pellets were resuspended in 0.5 ml of the same buffer and the nonphotoreactive cross-linking agent disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL), dissolved in dimethyl sulfoxide, was added to give a final concentration of 500 μM . The tubes were then incubated for 15 min in ice. To terminate the cross-linking reaction, 3 ml cold 10 mM Tris-HCl buffer, pH 7.4, were added and the tubes were centrifuged at $3,000 \times g$ for 20 min. The pellets were solubilized with electrophoresis sample buffer (50 mM Tris-HCl, pH 7.4-2% sodium dodecyl sulfate-9% sucrose-0.005% bromophenol blue). The samples were boiled for 5 min in the presence of 100 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 5% polyacrylamide gel using the discontinuous buffer system of Laemmli (19). Gels were stained by 0.2% Coomassie Brilliant Blue R-250, dried, and exposed to Kodak X-ray film SAR-5 for 14 days at -70°C . The molecular weight of each band was determined using calibration kits for molecular weight determination (Pharmacia high molecular weight and low molecular weight calibration kits; Pharmacia, France). The marker protein was ferritin (M_r 220,000), phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), and lactate dehydrogenase (M_r 36,000).

Estradiol and Progesterone Receptor Assay. [^3H]-17- β -Estradiol (specific activity, 101 Ci/mmol) and [^3H]Org 2058 (specific activity, 53 Ci/mmol) were purchased from Amersham (Amersham-France, Paris, France); the non-radioactive diethylstilbestrol and cortisol were purchased from Steraloids, Inc. (Pawling, NJ) and the cold Org 2058 was from Amersham.

Both ER and PgR were determined by the dextran-coated charcoal method (20, 21). Our laboratory is affiliated to the European Organization of Research and Treatment of Cancer which organizes quality controls of the assays (22). Tumors with more than 10 fmol/mg protein ER were considered positive and tumors with more than 10 fmol/mg protein PgR were considered positive.

Statistical Analysis. The distributions of the parameters (ER, PgR, IGF1-R) were previously studied. They were log (ln) normal after excluding zero values, confirming what we had found in other studies (23).

Relations between variable were determined using a qualitative linked parameter test (χ^2 test). Correlations between parameters were assessed according to the Spearman *R* nonparametric test; moreover in order to explicit these correlations, linear regressions were performed on subsets of the statistical population.

RESULTS

Characterization of IGF1 Receptors in Biopsies. The Scatchard analysis (24) of the binding data of saturation of a pool of breast cancer membranes with increasing concentration of ^{125}I -IGF1 is shown Fig. 1. The Scatchard transformation demonstrates one class of high affinity binding sites ($K_d = 1 \text{ nM}^{-1}$). Fig. 2 illustrates the growth factor binding specificity in the same pool of breast cancer membranes. Native IGF1 and recombinant IGF1 have the same potency. Native IGF2 was able to compete for binding; insulin competes also for binding with

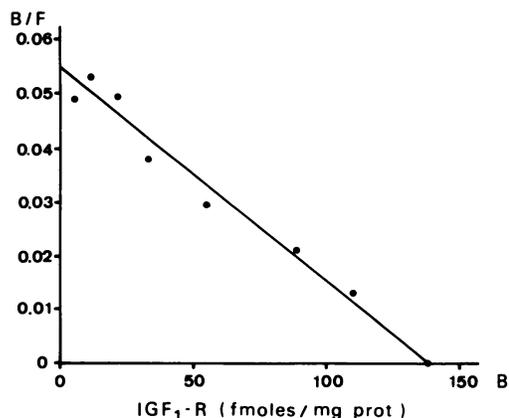


Fig. 1. Scatchard analysis performed by transformation of binding data from the saturation of pooled breast cancer membrane proteins (400 μg) with increasing concentrations of ^{125}I IGF1 ($K_d = 1 \text{ nM}^{-1}$; $N = 139 \text{ fmol/mg protein}$). *B/F*, bound versus free.

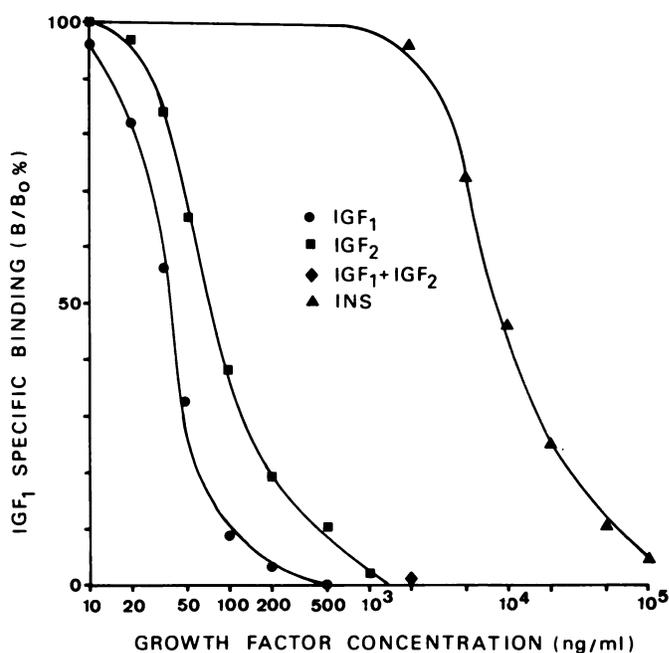


Fig. 2. Growth factor specificity of labeled IGF1 to pooled breast cancer membranes. Competition for binding (*B*) of ^{125}I [100% = binding in the absence of unlabeled growth factor (B_0)] in the presence of increasing amounts of unlabeled growth factors. *INS*, insulin.

a potency lower than 1/100 that of IGF1. Two μg of the crude preparation of IGF1-IGF2 completely inhibits the binding of labeled IGF1.

Results of chemical cross-linking of ^{125}I -labeled IGF1 on breast cancer membranes are presented in Fig. 3. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, the pool of breast cancer membranes and individual breast cancer membranes showed one band with a relative molecular weight of 130,000. Fig. 3 shows that a similar band is detected using our standardized breast cancer cell (BT-20) membrane preparation.

IGF1 Receptor Levels. Fig. 4 shows that 93% (71 of 76) of the tumors bound significantly more than 1% of the total labeled IGF1 and therefore were considered as IGF1-R positive. The specific binding ranged from 0 to 16.4%. The distribution was ln in an IGF1-R-positive tumor population; the geometric mean of IGF1-R positive was 3.9% ($3.4\text{--}4.6 \pm 2 \text{ SEM}$).

Steroid Receptors. ER was found in 83% (62 of 75) and PgR

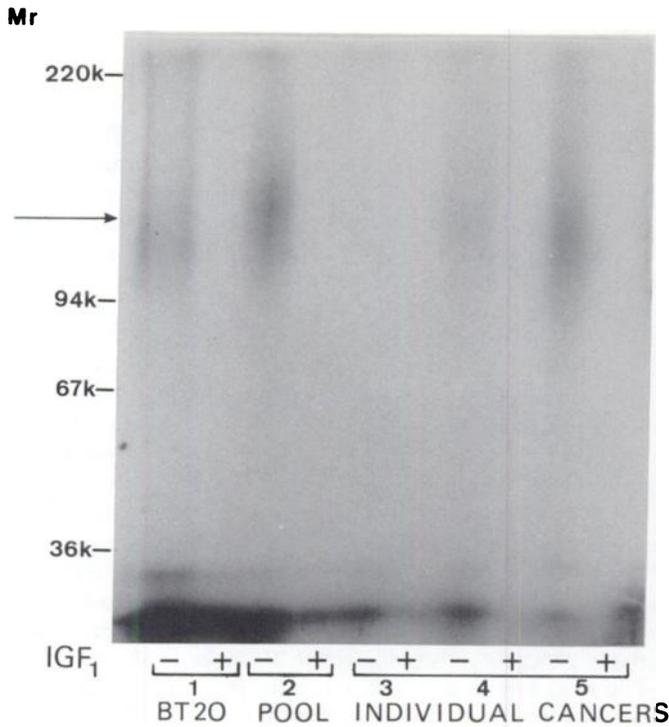


Fig. 3. Autoradiogram showing the size of ¹²⁵I-IGF1 binding unit complex in breast cancer membranes as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Membrane proteins (800 μg) were incubated with ¹²⁵I IGF1 in the absence (-) and in the presence (+) of unlabeled IGF1 (4 μg/ml). Cross-linking was performed with disuccinimidyl suberate (500 μM). The radioligand-binding site complexes were solubilized with sodium dodecyl sulfate containing dithiothreitol and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Arrow, position of M_r 130,000 (130 K) complexes. 1, BT-20 breast cancer cell membranes; 2, pooled breast cancer membranes; 3, 4, 5, individual breast cancer membranes. Ordinate, molecular weight in thousands.

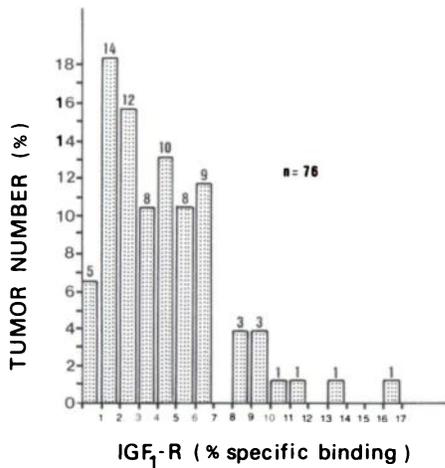


Fig. 4. Distribution of 76 human breast cancers as a function of their IGF1-R levels (in percentage of total counts/400 μg of membrane protein).

in 72% (54 of 75) of the tumors when the characteristics of positivity defined above are considered.

Relation between IGF1-R and Clinical Features. Considering the tumor type, only 4 of 61 ductal carcinomas and 1 of 9 lobular carcinomas were negative and all other types were positive. The mean level of IGF1 in ductal carcinomas was 4.09% (3.49–4.80 ± 2 SEM) and 3.2% (2.2–4.71 ± 2 SEM) in lobular carcinomas; the difference between these two mean levels was not statistically significant. In the lobular carcinoma the specific binding was 9.8%, in the colloid carcinoma it was

13.8%, in the two apocrine carcinomas it was 1.5 and 6.3% and in the two medullary carcinomas it was 1.6 and 1.8%.

In our population no relation was found between IGF1-R and histoprognostic grading according to the studies of Scarff and Bloom, cellular density, stromal reaction, and node metastases.

IGF1-R positivity rate was higher in postmenopausal patients ($\chi^2 = 6.779$; $P = 0.009$). The mean level of IGF1-R was 4.3% (3.7–5.1 ± 2 SEM) in postmenopausal patients and 3.3% (2.3–4.6 ± 2 SEM) in premenopausal ones.

Relation between IGF1-R and ER or PgR. There was no relation (χ^2 test) between IGF1-R and ER positivity rates in the whole population; conversely there was a strong relation between IGF1-R and PgR positivity rates ($P = 0.002$). Fig. 5 shows that IGF1-R levels were significantly higher ($P = 0.02$) in ER+ than in ER- tumors. The IGF1-R level in PgR+ tumors was 4.2% (3.6–5 ± 2 SEM); it was lower, but not significantly, than in PgR-negative tumors: 3.2% (2.3–4.5 ± 2 SEM). The

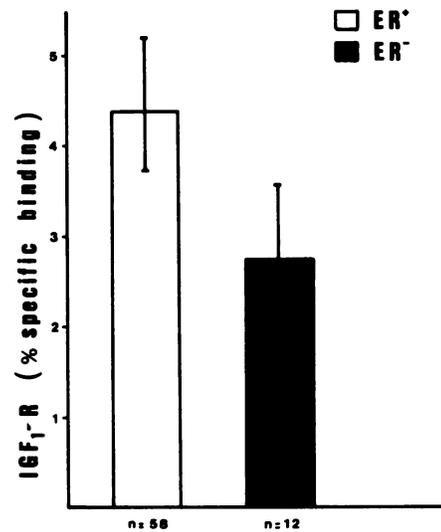


Fig. 5. Mean value ± 2 SEM (bars) of IGF1-R according to the ER status of the patients.

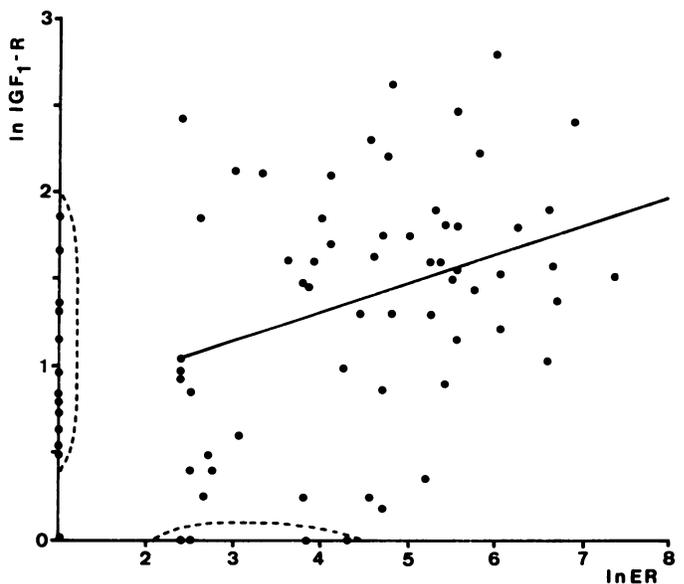


Fig. 6. Distribution of ln IGF1-R as a function of ln ER. Three groups of value could be noted. When zero values were excluded, a statistically significant positive linear correlation could be found between ln IGF1-R and ln ER ($r = 0.30$; $n = 58$; $P = 0.024$; $\ln \text{IGF1-R} = 0.154 \ln \text{ER} + 0.674$).

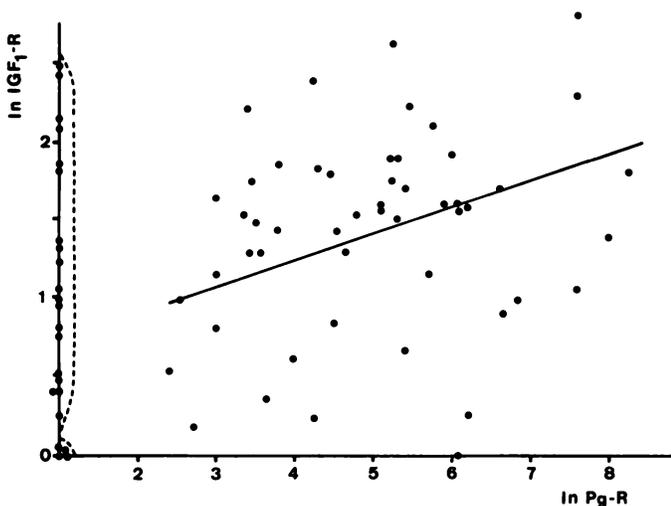


Fig. 7. Distribution of \ln IGF1-R as a function of \ln PgR. Three groups of value could be noted. When zero values were excluded, a statistically significant positive linear correlation could be found between \ln IGF1-R and \ln PgR ($r = 0.41$; $n = 53$; $P = 0.0025$; \ln IGF1-R = $0.169 \ln$ PgR + 0.545).

IGF1-R level was higher in ER+ PgR+ tumors: 4.5% (3.7 – 5.3 ± 2 SEM) than in ER– PgR– tumors: 2.7% (1.9 – 3.7 ± 2 SEM) ($P = 0.02$).

A correlation was found between IGF1-R and ER ($P = 0.0018$) and between IGF1-R and PgR ($P = 0.0011$) by the Spearman test.

However, these relations were complex as shown in Figs. 6 and 7. By representing \ln (IGF1-R) as a function of \ln (ER) (Fig. 6) we found that three populations of results could be individualized: population 1, zero values of ER; population 2, zero values of IGF1-R; population 3, nonzero values of both ER and IGF1-R. Therefore we must limit the study of a linear correlation to that third population. A statistically significant linear positive correlation was found between \ln ER and \ln IGF1-R ($r = 0.30$; $n = 58$; $P = 0.024$). The bidimensional representations of \ln IGF1-R as a function of \ln PgR showed, as previously, that 3 populations could be individualized. The study of correlation was performed in the population of nonzero values of PgR and IGF1-R; a statistically significant positive correlation was found between \ln PgR and \ln IGF1-R ($r = 0.41$; $n = 53$; $P = 0.0025$).

DISCUSSION

Research on IGF1-R has been hampered by the presence of a secreted, non-receptor binding protein; Clemmons *et al.* (25) have demonstrated that human fibroblast monolayers secrete a protein that binds IGF1, altering the IGF1-R binding.

In the present paper we demonstrate that IGF1 binds to membrane preparations from various tumors and that the binding is saturable, is specific, and corresponds to only one class of high affinity sites. Cross-linking experiments visualize one major band which corresponds to the expected size of the IGF1-R binding subunit. These results suggest that membrane preparations are devoid of non-receptor binding protein interference.

Almost all the human breast cancers that we studied contained IGF1 receptors (71 of 76). These findings suggest that most of the tumors could respond to IGF1 acting via either the endocrine, autocrine, or paracrine pathways.

Further studies would be useful to determine the exact tissular localization of IGF1-R in breast tumor; we cannot exclude the

possibility that IGF1-Rs are present on normal cellular components of the tumor tissue like fibroblasts (7). However, indirect evidence suggests that the majority of IGF1-R might be on the epithelial cells: (a) it has been demonstrated that human breast cancer cell lines are rich in IGF1-R (3); (b) IGF1-Rs are low in benign breast diseases and undetectable in normal breasts while these tissues contain a large fibroblastic compartment (26). The fact that IGF1-R in breast cancers was significantly increased compared to normal tissue or benign breast disease (26) suggests that the expression of IGF1-R is associated with the malignant transformation of breast epithelial cells.

We showed, using the Spearman test, that IGF1-R and ER or PgR were linked in breast cancers (15). In the present study, a more detailed analysis shows that the positivity of IGF1-R was associated to the positivity of PgR; this relation did not exist between ER and IGF1-R. Conversely IGF1-R levels were significantly higher in ER-positive than in ER-negative tumors; it was not the case with PgR. These results are corroborated by our observation of high IGF1-R in postmenopausal patients' breast cancer, already known to be rich in steroid receptors (23). Finally a linear correlation was found between ER and IGF1-R and between PgR and IGF1-R; these results are in line with the paper recently published by Pekonen *et al.* (27).

The mechanisms by which IGF1-Rs are regulated in breast cancer are poorly understood. The observed coexpression of IGF1-R and steroid receptors could suggest a common regulator for these receptors. *In vitro*, it has been demonstrated that human breast cancer cell lines devoid of ER (ER negative) express and secrete higher amounts of IGF1 than do ER-positive lines (11). It has also been found that IGF1-R concentration is higher in ER-positive lines than in ER-negative lines (8). These results suggest that the lower IGF1-R concentration in ER-negative lines and ER-negative tumors could be due to an occupation of the receptors by endogenous ligand or receptor down-regulation; it is possible that a similar process occurs in breast cancer tissue.

In conclusion, the present study confirms that IGF1 could be an important regulator of human breast cancer growth and may indicate that lowering the IGF1 serum concentration or IGF1-R could be a beneficial treatment of breast cancer.

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