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DEMONSTRATION OF INSULIN-LIKE GROWTH FACTOR (IGF-I AND -II) RECEPTORS AND BINDING PROTEIN IN HUMAN BREAST CANCER CELL LINES

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SUMMARY: The insulin like growth factors (IGFs), potent mitogens for a variety of normal and transformed cells, have been reported to be secreted by several human breast cancer cell lines (BC). We have investigated the binding characteristics of IGF-I and -II in four human BC: MCF-7, T-47D, MDA 231 and Hs578T. Binding studies in microsomal membrane preparations detected high specific binding for both IGF in all four BC studied. Cross-linking with ^{125}I-IGF-I, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions, revealed the presence of an α subunit of apparent M_r = 130,000 in MCF-7, T-47D and MDA 213 cells. When ^{125}I-IGF-II was cross-linked, a major band of apparent M_r = 260,000 was seen in all BC. This band was inhibited by IGF-II, but not by insulin. Cross-linking of ^{125}I-IGF-I to conditioned media from BC demonstrated the presence of three binding proteins of apparent M_r = 45,000, 36,000 and 29,000 in all BC but T-47D, in which the 36,000 band was not seen. These data demonstrate that BC possess classical receptors for both IGF-I and -II and, furthermore, that BC produce specific binding proteins for these growth factors.

Insulin-like growth factors (IGF-I and -II) are potent mitogens for a number of normal and transformed cell lines. The high degree of structural homology between the two IGFs results in crossreactivity for each other's receptors, as well as similar biological effects on target tissues (1). Nevertheless, IGF-I binds preferentially to a heterotetrameric type 1 receptor, while IGF-II binds preferentially to a monomeric type 2 receptor (2,3)

Recent studies have demonstrated that the IGFs may also be involved in the proliferation of human breast cancer cells (BC)(4-6). Specific IGF-I receptors have been demonstrated in 4 BC and they appear to be structurally identical to the IGF-I receptors identified in other tissues and both normal and transformed cell lines (7). Several BC have been reported to secrete IGF-I and to respond to stimulation by exogenous IGF-I in vitro (5,7).

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Furthermore, tumor growth in nude mice implanted with MCF-7 increased significantly when animals were treated with IGF-I (8). In these cells, IGF-I secretion is increased by estrogen, suggesting that the IGFs may act as estrogen-induced second messengers in estrogen-responsive BC (8).

To further investigate the possible role of the IGFs in the proliferation of BC, we utilized affinity cross-linking techniques to study IGF-I and -II receptors in two estrogen-dependent BC, MCF-7 and T-47D, and in two estrogen-independent BC, Hs578T and MDA 231. We also analyzed the media of these cells by cross-linking techniques to investigate the production of specific binding proteins for these growth factors by BC.

MATERIALS AND METHODS

Peptides: Pure biosynthetic (Thr-59)-IGF-I was purchased from Amgen Biologicals (Thousand Oaks, Ca). Pure synthetic IGF-II was generously provided by Dr. C.H. Li (San Francisco,CA). Recombinant DNA-derived human insulin was obtained from Eli Lilly & Company (Indianapolis, IN). Iodination was performed by a modification of the chloramine-T technique, to specific activities of 150-250 uCi/ug for both IGF-I and II. For determination of nonspecific binding, we employed a partially purified, insulin-free IGF preparation, containing both IGF-I and -II.

Culture of Breast Cancer Cells: MCF-7, MDA 231 and Hs578T cells were obtained from Dr. Adeline Hackett at the Peralta Cancer Research Institute (Oakland, CA). T-47D cells were obtained from Dr. Ricardo Tejada at Columbia University (New York). Cells were grown in DMEM supplemented with 4 mM L-glutamine, penicillin-streptomicin (100 ug/ml), 1 ug/ml insulin and 10% fetal calf serum. Cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide, at 37 C. HEP G2 cells were kindly provided by Dr. A.D. Cooper (Stanford University) and were maintained as described previously (9).

Preparation of Microsomal Membranes from BC: Confluent cell monolayers were detached by the addition of PBS, 1 mM EDTA. The 40,000g microsomal membranes were prepared, as previously described (10).

Charcoal Assay for Binding Proteins: Cells were grown as described above and when they were at least 80% confluent, cells were washed twice in PBS and incubated in serum free media (SFM), 0.1% BSA, for a period of 48 hours. Media were then collected, centrifuged at 500g for 10 minutes and the supernatant frozen at -20 C until assay by the charcoal separation method, as previously described (9).

Receptor Binding Assays; Affinity Cross-Linking and SDS-PAGE: Membranes were resuspended in 50 mM Tris HCl, 0.5% bovine serum albumin (BSA), pH 7.4. 50 ug of membrane protein were incubated with 10,000 cpm (binding assays) or 450,000 cpm (cross-linking) of iodinated peptide in the presence or absence of unlabeled peptide in a final volume of 500 ul for 2 hours at room temperature. After

incubation, 2 ml of Tris buffer were added, and samples centrifuged at 9000g for 30 minutes at 4C. The supernatant was discarded and the pellet counted in a gamma counter for determination of membrane-bound radioactivity, or resuspended in 1 ml Tris buffer without BSA. At this point the radioligand was cross-linked with 0.2 mM disuccinimidyl suberate (DSS) at 4C for 15 minutes. The reaction was quenched by the addition of 200 ul of 100 mM Tris, 10 mM EDTA, pH 7.4 and centrifuged at 9000g for 30 minutes at 4C. The pellet was resuspended in 50 ul of 2% SDS, pH 7.0, with 10% glycerol and 5% v/v 2-mercaptoethanol (sample buffer). Samples were boiled and electrophoresed on a 6% separating gel. For cross-linking of BC conditioned media, 10 ul of BC media were incubated with 50,000 cpm of iodinated peptide in the presence or absence of unlabeled peptide in a final volume of 50 ul at 4C overnight. Following incubation, 5 ul of 0.2 mM DSS were added at 4C for 15 minutes. The reaction was quenched with the addition of 30 ul of 100 mM Tris-EDTA, as described earlier. 50 ul of sample buffer were added and the preparation boiled for 5 minutes prior to electrophoresis on a 6-12.5% gradient gel.

RESULTS AND DISCUSSION: Table 1 shows the results of IGF-I binding to conditioned media from all 4 BC. The estrogen-dependent cells, T-47D and MCF-7, showed the greatest specific binding (24.5 and 19.5%, respectively), while Hs578T and MDA 231 binding were 2-4 fold less. The structures of these binding proteins were further characterized by cross-linking techniques. Figure 1 shows the results of SDS-PAGE of ^{125}I-IGF-I cross-linked to conditioned media from all 4 BC. Three bands of apparent M_r = 45,000, 36,000 and 29,000 (after substraction of IGF-I molecular weight) were seen in Hs578T (lanes 1-2) and MDA 231 (lanes 9-10) conditioned media. A major band (36-43,000) was prominent in T-47D (lanes 3-4) and MCF-7 (lanes 7-8) conditioned media and to a lesser extent, a minor band (45,000) was also seen in media from these cells. All three bands were inhibited by excess IGF. HEP G2 conditioned media

Table 1: Summary of ^{125}I-IGF-I and -II binding to conditioned media and membranes of human breast cancer cells

	% Specific binding				
	Conditioned Media*	BC Membranes**			
	IGF-I	IGF-I		IGF-II	
			+Ins		+Ins
MCF-7	19.5	8.8	2.1	9.7	8.4
T-47D	24.5	4.4	0.0	7.2	5.5
Hs578T	13.5	12.1	10.6	12.2	12.1
MDA 231	6.5	4.1	1.4	-	-

* % Specific binding to 50 ul of conditioned media, run at a 1:20 dilution.
** % Specific binding to 50 ug of microsomal membrane protein.
-: not assayed

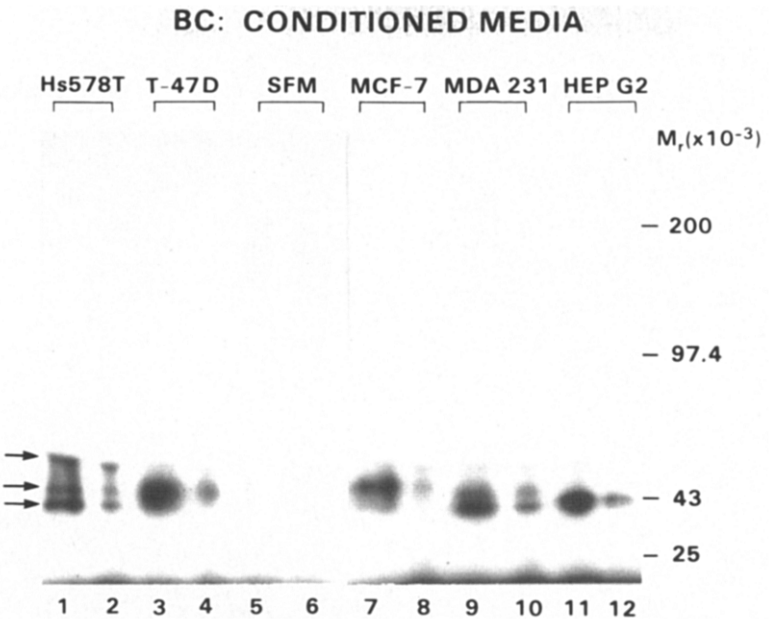

Figure 1: SDS-PAGE (6-15%) under reducing conditions of ^{125}I-IGF-I cross-linked to BC conditioned media from Hs578T (lanes 1-2), T-47D (lanes 3-4), unconditioned (SFM) media (lanes 5-6), MCF-7 (lanes 7-8), MDA 231 (lanes 9-10) and HEP G2 (lanes 11-12). Media were incubated in the presence (lanes 2,4,6,8,10,12) or absence (lanes 1,3,5,7,9,11) of excess unlabeled IGF.

(lanes 11-12) showed a major band of apparent M_r = 29,000 and a minor band of apparent M_r = 45,000.

Table 1 also shows the results of IGF-I and -II binding to membranes of all 4 BC. Specific binding of IGF-I was detected in all BC, and was highest in Hs578T, while MDA 231 showed relatively low binding. Binding of ^{125}I-IGF-I was displaceable by insulin, as well as IGF, in MCF-7, T-47D and MDA 231. Interestingly, IGF-I binding to Hs578T cells was poorly displaced by insulin, suggesting that the IGF-I binding was to an atypical IGF receptor or an IGF-binding protein. IGF-II binding was detected in all BC tested.

^{125}I-IGF-I cross-linking studies to all BC, except Hs578T, demonstrated a major band of apparent M_r = 130,000 (Figure 2). This 130,000 band was inhibited by unlabeled IGF and insulin, indicating that it represents the α subunit of the type 1 IGF receptor. A second band of apparent M_r = 230-250,000 was also inhibited by insulin and IGF-I, and probably represents incompletely reduced α-α dimers of the type 1 receptor. No specific bands were detected in Hs578T, supporting the previous

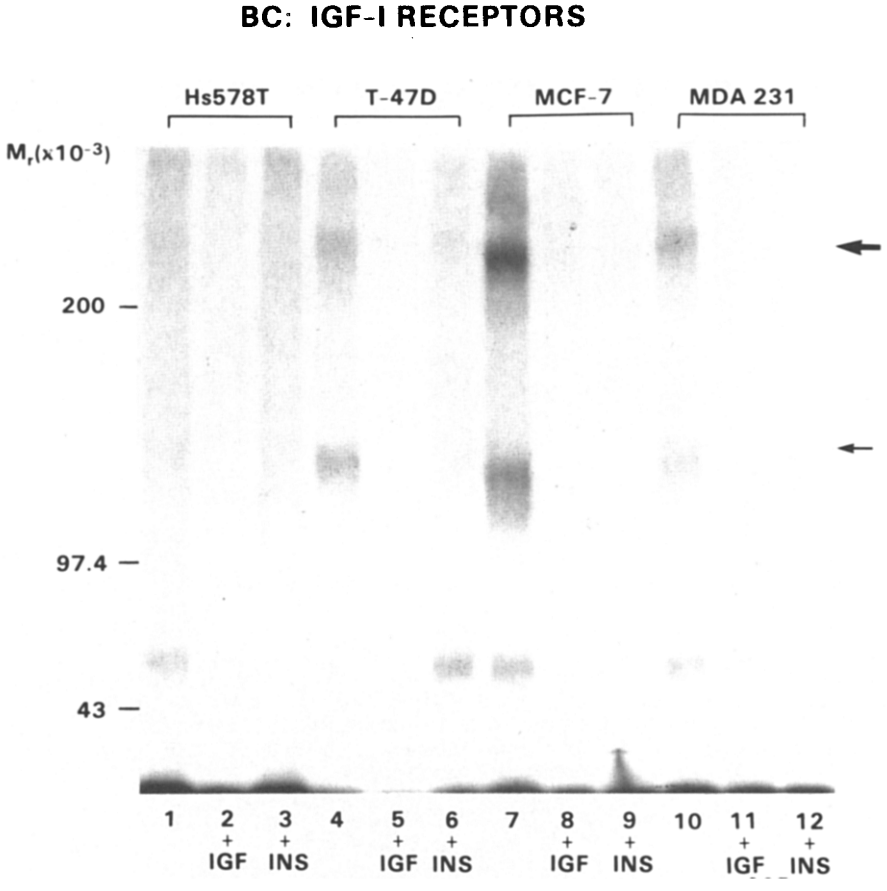

Figure 2: SDS-PAGE (6%) under reducing conditions of ^{125}I-IGF-I cross-linked to membranes from Hs578T (lanes 1-3), T-47D (lanes 4-6), MCF-7 (lanes 7-9) and MDA 231 (lanes 10-12). Incubations of BC membranes was performed in the absence of competing IGF (lanes 1,4,7,10) or in the presence of excess unlabeled IGF (lanes 2,5,8,11), or insulin (100 ug/ml; lanes 3,6,9,12).

observation that IGF-I is probably binding primarily to an IGF binding protein (see above), rather than a type 1 receptor. Figure 3 shows that cross-linking of ^{125}I-IGF-II to BC membranes results in a major band of apparent M_r = 260,000. This band was inhibited by unlabeled IGF-II (lanes 2,5,8,11), but not by insulin (lanes 3,6,9,12). This pattern of IGF-II binding is typical of the type 2 receptor. A second band of apparent M_r = 130,000 was also detected in T-47D, MCF-7, and MDA 231, but not Hs578T (lanes 4,7,10). This band was inhibited by excess IGF (lanes 5,8,11) and also by insulin, (lanes 6,9,12), and probably represents IGF-II cross-linked to the α subunit of the type 1 receptor. The fact that this second band is not present in Hs578T cells further supports our observation that IGF-I receptors in these cells are not present, or are too low to be detected.

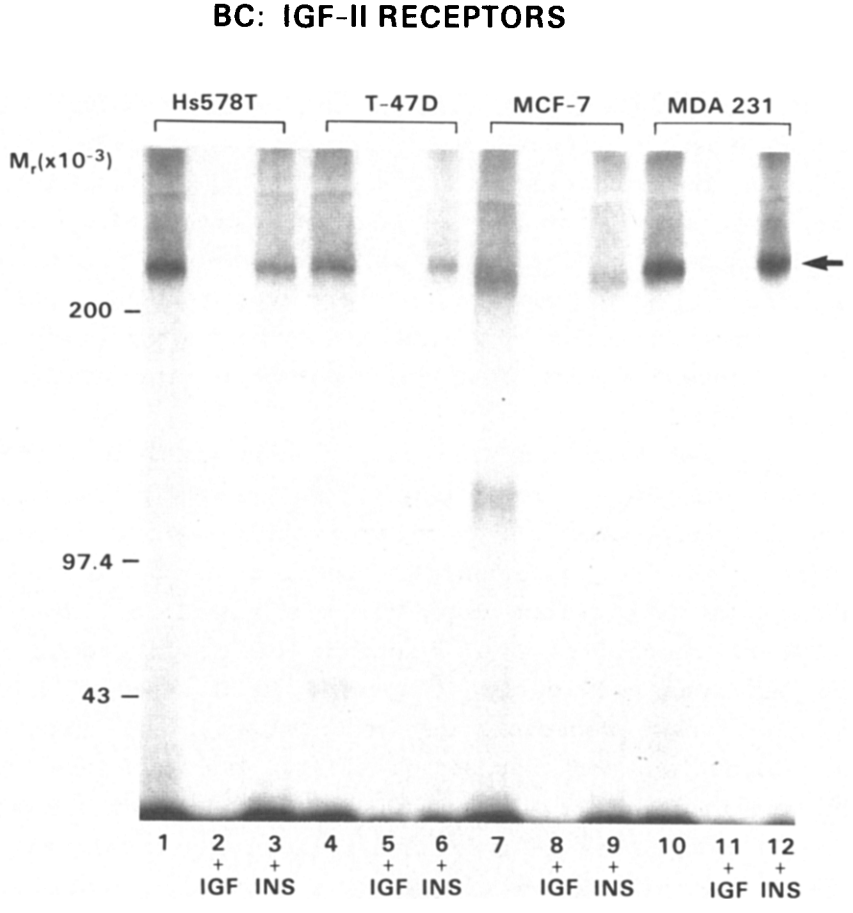

Figure 3: SDS-PAGE (6%) under reducing conditions of ^{125}I-IGF-II cross-linked to membranes from Hs578T (lanes 1-3), T-47D (lanes 4-6), MCF-7 (lanes 7-9) and MDA 231 (lanes 10-12). Incubations were performed in the absence of competing IGF (lanes 1,4,7,10) or in the presence of excess unlabeled IGF (lanes 2,5,8,11), or insulin (100 ug/ml; lanes 3,6,9,12).

The role of IGF-I and -II and their receptors in the proliferation of human breast cancer cells remains poorly understood. It has been reported that several BC secrete IGF-I, and that IGF-I and high concentrations of insulin stimulate DNA synthesis in some BC (7). Furthermore, Rohlik et al have recently shown that a monoclonal antibody directed against the type 1 IGF receptor inhibits replication of MCF-7 cells grown in 5% serum, suggesting that IGF-I may function as an important paracrine or autocrine growth factor for BC (11). Our studies indicate that BC also produce specific IGF binding proteins, which may play important roles in modulating IGF receptor binding and action (12,13). Similar binding proteins have been found in conditioned media from a variety of normal and transformed cells (14-16) and

recently in BC (17). The relationship of the BC binding protein(s) to these other proteins remains to be established. In addition to influencing IGF binding and action, these binding proteins may result in erroneous estimations of IGF-I and -II activity in unextracted samples (17-18). Preliminary studies from our laboratory, using unextracted BC conditioned media, indicate substantial apparent IGF-like activity, much of which can be accounted for by the presence of binding proteins. Following removal of these binding proteins by chromatography in formic acid, media concentrations of IGF-I have been less than 5 ng/ml (unpublished data).

Our studies also comfirm previous observations related to the presence of IGF-I receptors in BC, but point out the importance of accounting for binding protein before attributing binding activity to a specific receptor (as in Hs578T). Additionally, we have demonstrated the presence in all four BC of typical IGF-II receptors. The existence of other growth factor receptors has been previously documented in BC, and it has been suggested that these receptors may be involved in the progression of tumor formation and metastasis (19). The presence of IGF receptors and binding proteins in these cells indicates that both IGF-I and -II could play an important role in the proliferation of BC. Current studies are in progress to further investigate the nature of the IGF binding proteins in these cells and to study the biological actions of IGFs in BC proliferation and tumor formation.

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