Insulin-Like Growth Factor-II (IGF-II): A Potential Autocrine/Paracrine Growth Factor for Human Breast Cancer Acting via the IGF-I Receptor

C. Kent Osborne, Ester B. Coronado, Libbey J. Kitten, Carlos I. Arteaga, Suzanne A. W. Fuqua, K. Ramasharma, Milton Marshall, and Choh Hao Li†

Department of Medicine
Division of Oncology
University of Texas Health Science Center (C.K.O., E.B.C., L.J.K., C.L.A., S.A.W.F., M.M.)
San Antonio, Texas 78284

Laboratory of Molecular Endocrinology
University of California (K.R., C.H.L.)
San Francisco, California 94143

Insulin-like growth factor-II (IGF-II) is a potent mitogen for several types of cultured cells and tissues. We have studied the interaction of IGF-II with a panel of cultured human breast cancer cell lines, examining the possibility that these cells synthesize and secrete IGF-II activity which could have autocrine/paracrine functions. Synthetic IGF-II was mitogenic in five of seven cell lines tested, including the estrogen receptor-positive lines MCF-7L, ZR75-1, and T47D and the estrogen receptor (ER)-negative lines Hs578T and MDA-231. IGF-II was slightly less potent than IGF-I in stimulating DNA synthesis in MCF-7I cells, an effect that paralleled its ability to compete for [^{125}I]IGF-I binding in these cells. Affinity labeling studies revealed that IGF-II could also compete for binding to the 130,000 mol wt α-subunit of the IGF-I receptor. A monoclonal antibody to the IGF-I receptor inhibited the mitogenic effects of IGF-II in MCF-7L and MDA-231 cells, suggesting that this receptor mediates the growth effects of IGF-II in these breast cancer cells. Using a RIA and a RRA, IGF-II-like activity was detected in conditioned medium extracts processed to remove IGF-binding proteins from several breast cancer cell lines, with the highest levels found in conditioned medium from MCF-7L and T47D cell lines. IGF-II mRNA transcripts in MCF-7L and T47D cells were identified by Northern blot analysis and were confirmed by RNase protection assay. IGF-II mRNA was increased by estrogen in MCF-7L cells. These data suggest that IGF-II is an important mitogen for certain breast cancer cells and that its effects are mediated via the IGF-I receptor. The ability of these cells to express IGF-II mRNA and secrete IGF-II activity into the culture medium further supports the hypothesis that IGF-II may have autocrine/paracrine as well as endocrine growth regulatory functions in human breast cancer. (Molecular Endocrinology 3: 1701–1709, 1989)

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INTRODUCTION

The insulin-like growth factors (IGFs) are a family of polypeptide hormones with functional and structural homologies to insulin. IGF-I, under GH control, appears to be a major regulator of somatic growth (1). The normal function of IGF-II remains obscure, although it is a potent mitogen for many cultured cells (2).

Experimental data suggest that insulin and the IGFs may be important regulators of breast cancer growth. Physiological concentrations of insulin and IGF-I stimulate cell proliferation in several human breast cancer cell lines (3–6). Several years ago Myal *et al.* (7) reported that purified rat multiplication-stimulating activity, which is similar to human IGF-II, stimulated the growth of T47D breast cancer cells. More recently, IGF-II purified from human plasma (8) and recombinant human IGF-II (4) were reported to stimulate the growth of MCF-7 and T47D cells.

Insulin and the IGFs have their own distinct cell surface receptors. Receptors for insulin and IGF-I are similar, each composed of two subunits and both possessing tyrosine kinase activity (9). The so-called IGF-II receptor is a single polypeptide without intrinsic kinase activity. Recently, the IGF-II receptor has been shown to be identical to the mannose-6′-phosphate receptor, which is involved in lysosomal enzyme pathways (9).

It is not clear whether the IGF-II/mannose-6′-phos-

phate receptor mediates the cellular effects of IGF-II. Considerable cross-reactivity exists among insulin, the IGFs, and their receptors in many different tissues, and a growing body of evidence suggests that the cell-proliferative effects of all three hormones are mediated predominantly via the IGF-I receptor (10–12). Human breast cancer cells have been shown to specifically bind insulin, IGF-I, and rat multiplication-stimulating activity (IGF-II) with high affinity (5, 7, 13). Circumstantial evidence suggests that insulin's mitogenic effects may be mediated through the IGF-I receptor (5), but the mechanism by which IGF-II stimulates growth has not been studied in human breast cancer.

Recently, it has been shown that human breast cancer cells have the capacity to synthesize and secrete a variety of growth factor-like activities, including the IGFs (14). Huff *et al.* (6, 15) have reported that human breast cancer cells express IGF-I mRNA and secrete an IGF-I-like activity into conditional medium. Furthermore, this activity is estrogen regulated in ER-positive cells. Yee *et al.* (8) have reported that one of nine cultured human breast cancer cell lines studied expressed IGF-II mRNA, although secretion of IGF-II-like activity into conditioned medium was not studied. IGF-II mRNA was also found in human breast cancer biopsy specimens, normal breast biopsies, and fibroblasts, suggesting that IGF-II might be produced by both breast cancer cells and stromal tissues.

In the present study we examined the biological activity and receptor binding of IGF-II and found it to be mitogenic for several breast cancer cell lines. IGF-II is slightly less potent than IGF-I, it binds with high affinity to the IGF-I receptor, and its growth effects are inhibited by an antibody to IGF-I receptor, suggesting that this receptor partially mediates the growth effects of IGF-II. Furthermore, confirming and expanding a previous study (8), we have detected IGF-II mRNA transcripts in breast cancer cell lines using both Northern analysis and ribonuclease protection assay, and we have identified secreted IGF-II-like activity in conditioned medium from these lines by both RIA and RRA.

RESULTS

Effect of IGF-II on Breast Cancer Cell Proliferation

Synthetic IGF-II (1 nM) significantly increased monolayer growth of MCF-7 breast cancer cells maintained in serum-free, phenol red-free tissue culture medium (Fig. 1). Control cells incubated in medium alone grew slowly over the 8-day culture period. Incubation with IGF-II resulted in more than a 4-fold increase in cell number by day 8. Thus, like insulin and IGF-I (5, 13), IGF-II is a potent mitogen for MCF-7L cells.

IGF-II growth effects were next assessed in a panel of both ER-positive and ER-negative breast cancer cell lines using the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material as an index of DNA synthesis and cell proliferation. IGF-II treatment

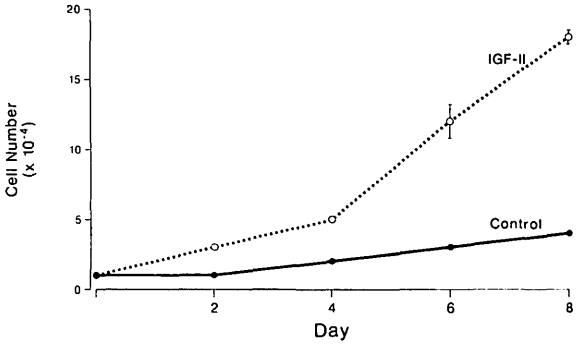

Fig. 1. Effect of IGF-II on MCF-7L Cell Proliferation

MCF-7L cells were plated in full medium with serum as described in *Materials and Methods*. Forty-eight hours later the medium was exchanged for serum-free, phenol red-free medium with IGF-II (1 nM) or vehicle alone (controls). Fresh medium was exchanged for spent medium every other day. Cell number was determined on the indicated days. *Points* represent the mean ± 1 SE of triplicate determinations.

Table 1. Effect of IGF-II on Thymidine Incorporation in a Panel of Human Breast Cancer Cell Lines

Cell Line	ER Status	[^{3}H]Thymidine Incorporation (cpms × 10^3)[a]	
		Control	IGF-II
MCF-7L	+	18.7 ± 0.4	100.1 ± 4.8[b]
ZR75-1	+	18.8 ± 1.2	22.8 ± 0.7[b]
T47D	+	11.1 ± 0.9	24.5 ± 2.0[b]
HS578T	−	0.9 ± 0.04	1.3 ± 0.1[b]
MDA-231	−	50.0 ± 0.5	69.6 ± 2.3[b]
BT20	−	15.1 ± 0.4	14.3 ± 0.5
MDA-330	−	7.5 ± 0.1	2.6 ± 0.2[b]

[a] Mean of triplicates ± SE.
[b] $P < 0.05$.

caused a statistically significant increase in [^{3}H]thymidine incorporation in five of the seven cell lines tested, including MCF-7L, ZR75–1, T47D, Hs578T, and MDA-231 (Table 1). The degree of stimulation varied among these cell lines and to some extent between experiments in an individual cell line; stimulation ranged from only 20–30% in the ZR75–1 cells to more than 3-fold in MCF-7L cells. The BT2O line was consistently unaffected by IGF-II under these conditions. Interestingly, the MDA-33O cell line was consistently inhibited by IGF-II.

To determine the relative mitogenic potencies of IGF-I and IGF-II, dose-response studies of [^{3}H]thymidine incorporation in MCF-7L cells were performed. In several experiments IGF-II was slightly less potent than IGF-I, as shown in Fig. 2 where the dose-response curve for IGF-II was shifted slightly to the right. Maximal stimulation was similar for both factors and was observed between concentrations of 1–10 nM.

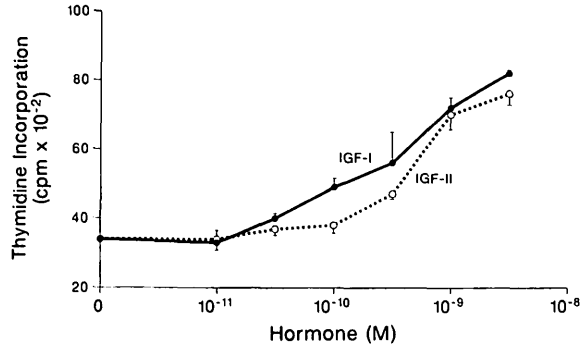

Fig. 2. Effects of IGF-I and IGF-II on DNA Synthesis in MCF-7L Cells

Hormones were added to cells growing in serum-free phenol red-free medium, and [^{3}H]thymidine incorporation into acid-precipitable material was determined as previously described (24). *Points* represent the mean ± 1 SE of triplicate determinations.

Binding of IGF-II to the IGF-I Receptor

To determine whether the effects of IGF-II are mediated through the IGF-I receptor, we first carried out competitive binding and affinity labeling studies. MCF-7L and MDA-231 cell lines were incubated with [^{125}I]IGF-I with increasing concentrations of unlabeled IGF-I, IGF-II, or insulin (Fig. 3). Both IGFs and insulin competed for IGF-I binding, although IGF-I was slightly more potent than IGF-II, and both IGFs were significantly more potent than insulin. Half-maximal displacement was observed with 2–3 nM IGF-I and 5–6 nM IGF-II. Insulin was 10-fold less effective in these competition studies.

Affinity labeling studies using disuccinimydil suberate (DSS) to chemically cross-link [^{125}I]IGF-I to MCF-7L cells followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis under reducing conditions revealed three strongly labeled bands with mol wt (Mr) of approximately 130,000, 220,000, and 330,000 (Fig. 4). Binding to the 130,000 Mr band, which probably represents monomers of the α-subunit of the IGF-I receptor, was inhibited significantly by IGF-I (1 nM), partially inhibited by IGF-II (1 nM), and minimally inhibited by the same concentration of unlabeled insulin. Binding to the larger species was also inhibited by IGF-I, IGF-II, and, to a lesser extent, insulin, indicating that they are probably incompletely reduced dimers or aggregates of IGF-I receptor subunits. These specificity studies suggest that IGF-II binds with high affinity to the IGF-I receptor in human breast cancer cells.

Effect of IGF-I Receptor Antibody Blockade on IGF-II-Induced Growth

To further investigate the importance of the IGF-I receptor in IGF-II-induced growth of human breast cancer cells, we used a monoclonal IGF-I receptor antibody (αIR_3) that blocks the hormone-binding domain of the receptor (16). MCF-7L and MDA-231 cells were incubated with increasing concentrations of IGF-II with or without αIR_3. Similar to its effect on IGF-I-induced growth, blockade of the IGF-I receptor with αIR_3 significantly inhibited IGF-II-induced growth (Fig. 5). Control antibodies (αIR_1 and 323A3) had no effect on cell proliferation (not shown). Furthermore, in other experiments the inhibitory effect of αIR_3 was reversible with excess IGF-I, indicating that it was not due to nonspe-

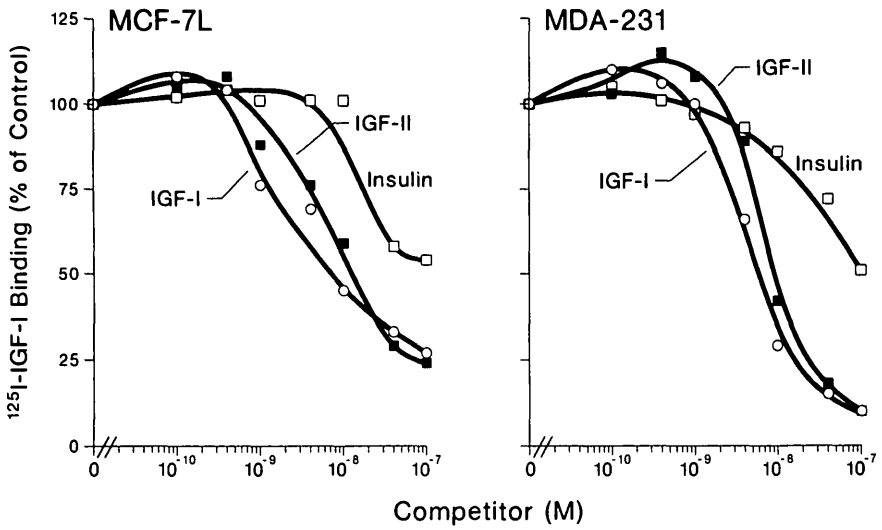

Fig. 3. Relative Potencies of IGF-I, IGF-II, and Insulin for the IGF-I Receptor in MCF-7L and MDA-231 Cells

Cells were plated as described in *Materials and Methods* and incubated with [^{125}I]IGF-I (40 pM) and the indicated concentrations of unlabeled IGF-I, IGF-II, or insulin for 3 h at 4 C. The monolayers were washed, and cell-associated radioactivity was determined. Under these conditions MDA-231 cells bound 6% and MCF-7 cells 8.5% of the total added radioactivity. *Points* represent the mean of triplicate determinations.

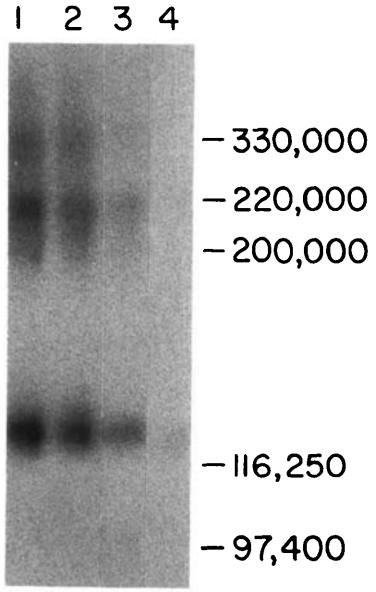

Fig. 4. Affinity Labeling of the IGF-I Receptor in MCF-7L Cells

Confluent monolayers were incubated with 400,000 cpm [^{125}I]IGF-I without (lane 1) or with insulin (lane 2), IGF-II (lane 3), or IGF-I (lane 4), all at a concentration of 1 nM, for 4 h at 4 C. After washing, DSS (0.1 mM) was added for 15 min, and the reaction was quenched with Tris buffer. The cells were solubilized, and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3–10% gradient) and autoradiographed.

cific toxicity by the antibody (our manuscript submitted). These results suggest that the growth effects of IGF-II on human breast cancer cells are mediated, at least partially, through the IGF-I receptor.

IGF-II Activity in Conditioned Medium

Conditioned medium from six breast cancer cell lines was analyzed for IGF-II acivity by RIA and RRA. To minimize the effect of somatomedin-binding proteins, known to be produced by these cells, which could interfere with the assay by competing for the [^{125}I]IGF-II tracer, media were first acidified to dissociate IGF-II from the binding proteins, and the two activities were then separated by a manual reverse phase technique using Sep-Pak C-18 cartridges (17). No detectable IGF-II activity was detected in unconditioned medium processed in this fashion using either assay. All of the cell lines had detectable IGF-II immunoreactivity and IGF-II receptor-binding activity in their conditoned media (Table 2). Four of the lines (BT20, ZR75–1, MDA-231, and MDA-330) contained very low levels of activity; two of the cell lines (T47D and MCF-7L), however, secreted significant amounts of IGF-II-like material into the culture medium. Conditioned medium from these two estrogen receptor-positive lines contained 4- to 300-fold more IGF-II activity than that from the other lines.

Northern Analysis of IGF-II mRNA

Three intense IGF-II bands of approximately 6.4, 4.6, and 0.3 kilobases (kb; Fig. 6) were revealed in MCF-7L and T47D cells by Northern blot analysis of poly(A)$^+$ mRNA. Five other breast cancer cell lines displayed only the 4.6- and/or the 0.3-kb bands. Interestingly, the relative intensities of these bands varied among cell lines. The high level of IGF-II mRNA in T47D cells is in agreement with the markedly increased IGF-II protein levels detected in the conditioned medium from these cells.

IGF-II mRNA expression was increased by estrogen treatment of MCF-7L cells (Fig. 7). The 6.4- and 4.6-kb mRNA transcripts, but not the 0.3-kb message were increased by treatment of MCF-7L cells growing in serum-free phenol red-free medium with 1 nM 17β-estradiol. The increased band intensity seen with estrogen was not due to differences in RNA loading or transfer, as shown by similar band intensity after rehybridization with β-actin. These results are similar to those previously reported in T47D cells (8). Thus, IGF-II expression appears to be regulated by estrogen in ER-positive human breast cancer.

Ribonuclease Protection Assays for IGF-II mRNA

To confirm the presence of genuine IGF-II mRNA in these cells solution hybridization of a labeled IGF-II RNA probe to total RNAs extracted from the cell line panel followed by treatment with RNase-A and -T1 were performed. Hybrid species formed by the probe reacting with authentic IGF-II mRNA are protected from the enzymatic digestion. Using this technique authentic IGF-II mRNA was detected again in the MCF-7L and T47D cell lines (Fig. 8). A faint band was also observed with RNA extracts from MDA-231 cells, but the ZR75–1, Hs578T, BT2O, and MDA-330 lines had no detectable IGF-II message in this assay, nor was a band visible in these cell lines when the total RNA in the assay was increased 2-fold. Estrogen stimulation of IGF-II in MCF-7 cells was also confirmed using the RNase protection assay (data not shown). Scanning densitometry revealed a 4- to 5-fold increase in IGF-II mRNA with estrogen.

DISCUSSION

We have demonstrated that IGF-II is a potent mitogen for some cultured human breast cancer cells using synthetic hormone which is devoid of possible IGF-I contamination with material purified from plasma. A proliferative response was obtained with IGF-II in five of seven cell lines tested, including both ER-positive and ER-negative cells. For unclear reasons, one cell

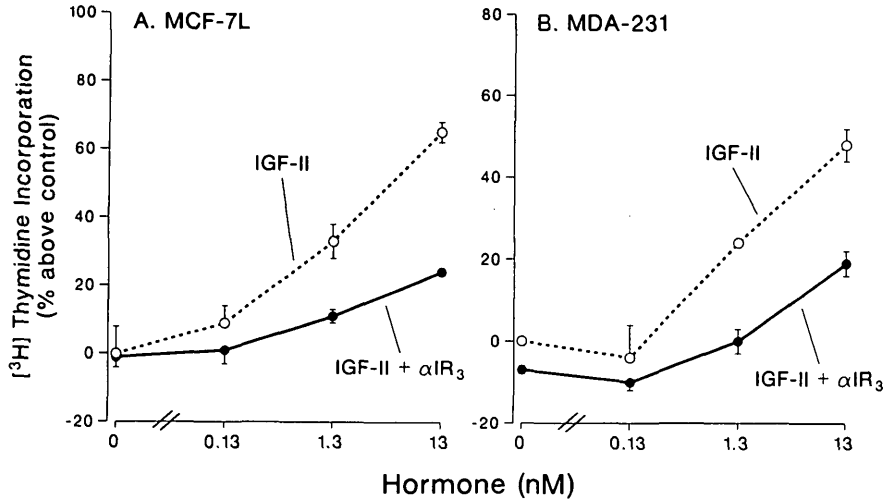

Fig. 5. Inhibition of IGF-II-Induced Growth by αIR_3 in MCF-7L and MDA-231 Cells

Cells growing in serum-free phenol red-free medium were incubated with IGF-II in the absence or presence of αIR_3 antibody (10 nM). [^{3}H]Thymidine incorporation was determined, as described above, in MCF-7L cells (A) and MDA-231 cells (B). *Points* represent the mean ± 1 SE of triplicate determinations.

Table 2. IGF-II Activity in Conditioned Media (CM) from Human Breast Cancer Cell Lines

Cell Line	Vol of CM (ml)	Total Cell No. ($\times 10^7$)	RIA		RRA	
			ng/ml[a]	ng/ml·10^7 cells	ng/ml[a]	ng/ml·10^7 cells
T47D	500	15.7	2125 ± 231	135.4	1750 ± 140	111.5
MCF-7L	500	76.1	360 ± 56	4.7	1100 ± 77	14.4
BT20	500	22.0	20 ± 3	.91	90 ± 5	4.1
ZR75-1	500	46.5	30 ± 5	.64	26 ± 4	.56
MDA-330	500	170.6	33 ± 3	.19	11 ± 3	.06
MDA-231	500	27.5	10 ± 2	.36	60 ± 5	2.2

[a] Mean of triplicates ± SD.

line, MDA-330, was inhibited by IGF-II. Nevertheless, IGF-II, like IGF-I and insulin (3–6), appears to be a growth factor for cultured breast cancer cells, confirming previous observations using purified (7, 8) or recombinant material (4).

Our data also suggest that the proliferative effects of IGF-II for breast cancer cells are mediated predominatly through the IGF-I receptor (type I somatomedin receptor). First, IGF-II was only slightly less potent than IGF-I in stimulting DNA synthesis, and its biological effects paralleled its ability to compete for the binding of labeled IGF-I to breast cancer cells. Second, as shown by affinity labeling studies, IGF-II bound to the 130,000 Mr α-subunit of the IGF-I receptor in these cells. Finally, antibody blockade of the IGF-I receptor markedly inhibited IGF-II-induced growth. These experiments do not exclude the possibility that some growth stimulation by IGF-II is mediated through the IGF-II/mannose-6-phosphate receptor. Recent reports suggest that Ca^{+2} influx in 3T3 fibroblasts (18), amino acid uptake in myoblasts (19), DNA synthesis in a leukemia cell line that expresses IGF-II, but not IGF-I receptors (20), and glycogen synthesis in hepatoma cells (21) are all stimulated by IGF-II acting through the IGF-II/mannose-6-phosphate receptor. Furthermore, a cathepsin-D-like protein which binds to this receptor is mitogenic for cultured breast cancer cells (22). In preliminary studies (unpublished data) we have found that a monoclonal antibody specific for the IGF-II receptor modestly stimulates DNA synthesis in MCF-7 cells. Nevertheless, the cumulative evidence in breast cancer cells as well as in other model systems (9–12) suggests that a major mediator for IGF-II-induced cell proliferation is the IGF-I receptor. This observation has potentially important clinical implications. Analogous to antiestrogen-induced growth inhibition achieved by blocking the ER, blockade of the IGF-I receptor by an antagonist might inhibit the growth stimulatory effects of three related hormones (IGF-I, IGF-II, and insulin), perhaps offering a new treatment strategy.

Yee and colleagues (8) recently have demonstrated IGF-II mRNA in T47D cells, human breast cancer specimens, and human fibroblasts. Our data confirm the high IGF-II mRNA levels in T47D cells, although we also detected IGF-II in MCF-7L cells (Yee also reported IGF-II mRNA in a late passage MCF-7 line). We found transcripts by Northern blot analysis hybridizing to our IGF-II cDNA probe in several other breast cancer cell

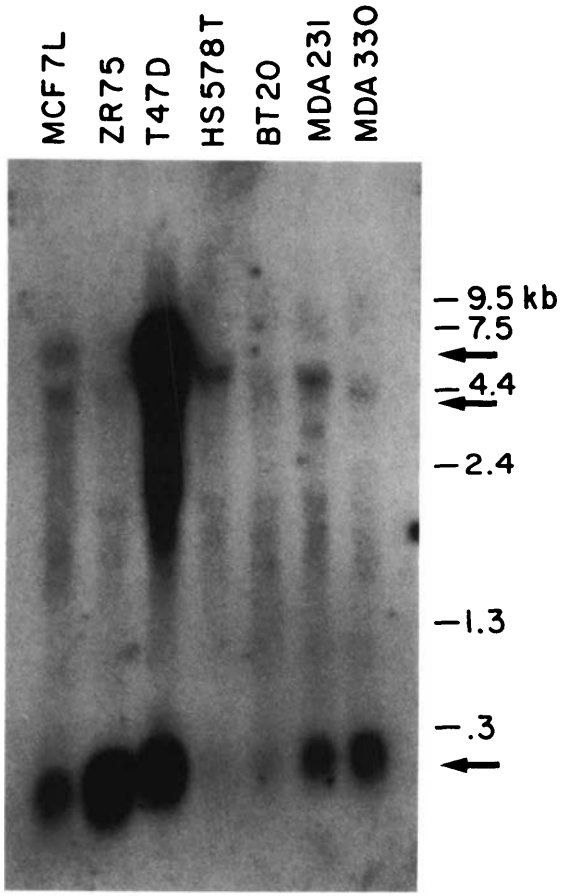

Fig. 6. Northern Blot Analysis of RNA Extracts from a Panel of Breast Cancer Cell Lines Probed with an IGF-II cDNA

Five micrograms of poly(A)$^+$ RNA were electrophoresed in a 1% agarose gel and then transferred to nitrocellulose as described in *Materials and Methods*. The RNA was hybridized with a ^{32}P-labeled IGF-II cDNA, washed, and autoradiographed.

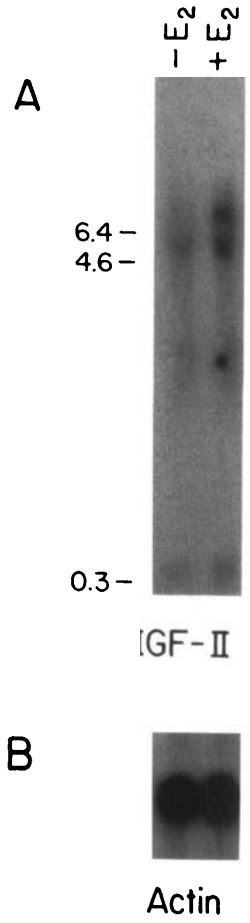

Fig. 7. The Effect of Estrogen on Accumulation of IGF-II mRNA in MCF-7L Cells

MCF-7L cells growing in serum-free phenol red-free medium were incubated with 1 nM 17β-estradiol (E_2) for 24 h. mRNA was isolated, electrophoresed, and transferred to nitrocellulose as described in the text, and the blot was probed with a ^{32}P-labeled IGF-II cDNA (A). To control for variable loading and transfer, the blot was washed and reprobed with B-actin (B).

lines, but these were not confirmed by the more sensitive and specific RNase protection assay. Whether these other bands detected with Northern analyses represent cross-reacting species is not clear and requires further analysis. Specifically, the origin of the 0.3-kb cross-reacting RNA seen by us and others (8) is currently unknown.

We have also found biologically active and immunoreactive IGF-II-like activity by RRA and RIA in conditioned medium extracts from several breast cancer cell lines. Consistent with the RNA studies, conditioned medium from MCF-7L and especially T47D contained significant quantitites of IGF-II-related material. The other cell lines tested secreted very low, but detectable, levels of IGF-II activity into their conditioned medium. It is possible that the low levels of activity in these lines represent authentic IGF-II-like activities, and that our RNA assays were too insensitive to demonstrate IGF-II mRNA in these lines. It is more likely that the low level activity represents residual contamination of IGF-binding proteins despite the extraction procedure. However, it is highly unlikely that the significant IGF-II-like activity in conditioned medium from MCF-7L and T47D cells is an artifact due to IGF-binding proteins. First, the conditioned medium was acidified, and the IGFs and binding proteins separated by a technique that has previously been shown to be effective in removing binding proteins (17). Second, in preliminary studies we have shown that all of the lines studied here secrete somatomedin-binding proteins, with MDA-330 and ZR75-1 containing the highest levels in their conditioned medium (Clemmons, D., and K. Osborne, manuscript submitted). The low level of IGF-II-like activity that we observed in this line suggests that most of the binding proteins have been removed, confirming that the IGF-II activity in MCF-7L and T47D conditioned medium was not due to residual binding proteins. Finally, specific IGF-II mRNA was detected by RNase protection assay in MCF-7L and T47D cells. Similar to the data reported by Yee *et al.* (8), DNA slot blots using an IGF-II probe showed no evidence of gene amplifica-

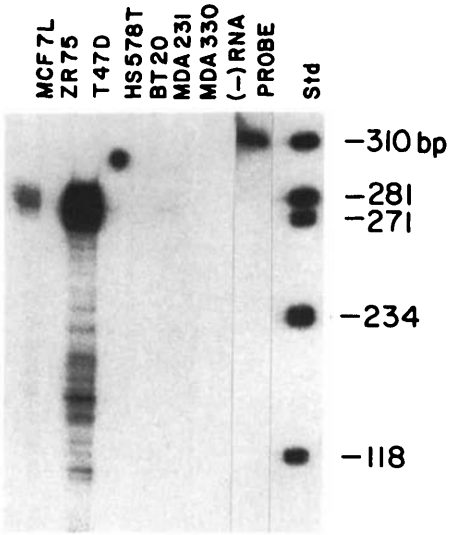

Fig. 8. RNase protection assay for IGF-II mRNA from a panel of human breast cancer cells. Forty micrograms of total RNA from each cell line were hybridized with an IGF-II RNA probe as described in *Materials and Methods*. Samples were digested with RNase, extracted with phenol/chloroform, precipitated, and electrophoresed. The gel was then dried and autoradiographed.

tion as a mechanism for the high IGF-II mRNA expression in T47D cells (data not shown).

In summary, certain cultured human breast cancer cell lines produce IGF-II, a growth factor that is also expressed in human breast cancer tissue specimens (8). IGF-II is also present in human plasma, and it is produced by tumor stromal tissues, such as fibroblasts. Furthermore, human breast cancer cell lines and breast cancer tissues (23) possess IGF-I receptors which mediate the growth effects of IGF-II. These cumulative data suggest that IGF-II may have important endocrine, paracrine, and autocrine functions in the growth regulation of human breast cancer.

MATERIALS AND METHODS

Cells, Hormones, and Antibodies

The human breast cancer cell lines MDA-231, MDA-330, Hs578T, ZR75-1, MCF-7L, BT20, and T47D were obtained and cultured as previously described (24). Cells were passaged weekly by first suspending them in trypsin-EDTA. Recombinant human IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Synthetic IGF-II was produced as described previously (25, 26). This material has been shown to have bioactivity equivalent to purified IGF-II (20). Porcine insulin was purchased from Sigma (St. Louis, MO). The αIR_3 and αIR_1 monoclonal antibodies were the gifts of Dr. S. Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC). The 323A3 antibody was provided by Dr. W. L. McGuire (San Antonio, TX). The αIR_3 antibody blocks IGF-I receptor binding (16). The αIR_1 recognizes a nonbinding domain of the human insulin receptor and is devoid of biological activity (27). The 323A3 antibody recognizes an irrelevant antigen on the surface of breast cancer cells (28). The αIR_1 and 323A3 antibodies served as controls and demonstrated no biological activity in these experiments.

Mitogenic Assays

The effect of hormones on cell proliferation was assessed in a monolayer growth assay. Cells were plated replicately in six-well dishes (Corning Laboratories, Houston, TX) at a low density (0.4×10^4 cells/dish) in improved minimal essential medium (IMEM; Irvine Scientific, Alamosa, CA) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY) and 10 nM insulin. Forty-eight hours later spent medium was exchanged for serum-free, phenol red-free IMEM supplemented with IGF-II (7.5 ng/ml or 1 nM) or vehicle alone as a control [PBS with 0.1% BSA (PBS/BSA)]. Fresh medium plus hormones were exchanged every other day for spent medium, and cell counts were determined after suspending the cells with 1 nM EDTA.

DNA synthesis was estimated by the incorporation of [^{3}H] thymidine into trichloroacetic acid-precipitable material as previously described (24), except that hormones were added to cells growing in phenol red-free, serum-free medium. The αIR_3 or control antibody was also added at the indicated concentrations.

Receptor Binding and Affinity Labeling Studies

[^{125}I]IGF-I (SA, 2125 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Cells were grown in 24-well tissue culture plates (Corning Laboratories, Houston, TX). When confluent, the monolayers were washed twice with binding buffer (IMEM, 0.1% BSA, and 25 mM HEPES, pH 7.4) and incubated with 40 pM [^{125}I]IGF-I in the absence or presence of various concentrations of unlabeled hormones for 3 h at 4 C (equilibrium binding conditions). After washing three times in ice-cold PBS-0.1% BSA, the cells were solubilized in 0.5 N NaOH. Cell-associated radioactivity was determined in a Analytic 1185 γ-counter with an efficiency of 78%.

For affinity labeling studies, confluent monolayers in six-well culture plates were incubated with 400,000 cpm [^{125}I]IGF-I without or with the specified concentration of unlabeled competitor hormone in 1 ml binding buffer for 4 h at 4 C. Monolayers were washed with cold PBS and then incubated with the cross-linking agent DSS (0.1 mM; Pierce Chemicals, Rockford, IL) in 2 ml HEPES buffer (120 mM NaCl, 5 mM KCl, 1.2. mM $MgSO_4$, 10 mM KH_2P0_4, and 20 mM HEPES, pH 7.4) for 15 min at room temperature. The mixture was aspirated, and the cross-linking reaction was quenched by adding 3 vol Tris buffer (10 mM Tris and 1 mM EDTA, pH 7.0). The cells were solubilized in 0.5 M Tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, and 0.001% bromophenol blue and boiled, and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3–10% gradient). The gels were stained with 0.25% Coomassie blue, destained with 7% acetic acid, and exposed to Kodak XAR-5 x-ray film (Eastman Kodak, Rochester, NY) at −70 C overnight.

IGF-II Activity in Conditioned Medium

Cells were grown to near confluence in T-150 flasks. After washing the monolayers three times with PBS-0.1% BSA, they were incubated in serum-free IMEM for 72 h at 37 C in a 5% $C0_2$ incubator. The media (500 ml/cell line) were then harvested, and aprotinin 0.2% (20 trypsin inhibitor units/ml; Sigma, St. Louis, MO) and pepstatin (1 μg/ml; Boehringer Mannhein, Indianapolis, IN) were added. The media were centrifuged to remove debris and then dialyzed against 30 liters 0.1% acetic acid at 4 C in Spectrapor 3 dialysis tubing (3500 Mr cut-off). The media were then lyophilized and reconstituted in 5 ml 0.1% acetic acid. To minimize interference in the RIA and RRA of somatomedin-binding proteins known to be secreted by these breast cancer cells, IGF activity was separated from the binding protein using Sep-Pak C-18 cartridges

(Waters Associates/Millipore, Inc., Milford, MA) as previously described (17). The IGF activity was eluted from the column in 50% acetonitrile, lyophilized, and then resuspended in PBS-0.01% acetic acid before analysis. Medium that had not been conditioned by cells was processed indentically and served as a control.

IGF-II activity in the processed conditioned medium was assayed in both a RIA and an RRA. Details of the RIA have been described previously (29).

Synthetic IGF-II was iodinated by the chloramine-T method. The rabbit polyclonal IGF-II antibody used in these experiments is specific for IGF-II, with minimal cross-reactivity with IGF-I (<6% cross-reactivity compared to IGF-II). The sensitivity of this RIA was 12 ng/tube. Dilutions of the conditioned medium extracts resulted in competition curves parallel to the standard curve in the IGF-II RIA for all cell lines tested.

The IGF-II RRA used BRL-3A cells, which have been shown to have specific IGF-II, but not IGF-I, receptors (30). These cells were plated in 24-well tissue culture plates. When nearly confluent the monolayers were washed three times with binding buffer and then incubated with [^{125}I]IGF-II (20,000 cpm/well) and increasing concentrations of unlabeled IGF-II (0.5–500 ng/ml) or aliquots of processed conditioned medium for 4 h at 4 C. The cells were washed three times with cold PBS-0.1% BSA and solubilized with 1 N NaOH. Cell-associated radioactivity was determined in a γ-counter. The sensitivity of the RRA was 5 ng/well.

RNA Isolation and Northern Hybridization

Total cellular RNA was extracted by homogenization in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion (31, 32). Poly(A)$^+$ RNA was prepared by oligo(dT) chromatography (Collaborative Research, Lexington, MA) (33). Five micrograms of RNA were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose. cDNA inserts of 811 and 215 basepairs (bp) were prepared by digestion with *Pst*I from the IGF-II higf2 clone kindly provided by Chiron Corp. (Emeryville, CA). Both fragments were labeled with ^{32}P by nick translation to a specific activity of 1×10^8 (33). Hybridization was carried out for 18 h at 50 C in 50% formamide-1 × Denhardt's-0.1% sodium dodecyl sulfate-100 μg/ml denatured salmon sperm DNA-5 × SSC with 5×10^6 cpm/ml. After hybridization, the membranes were washed with 0.2 × SSC-0.2% sodium dodecyl sulfate at 50 C and autoradiographed. To control for variable RNA loading and transfer, the IGF-II probe was removed from the nitrocellulose membranes, and the blots were rehybridized with a rat β-actin probe, kindly provided by Dr. Ellen Kraig.

Ribonuclease Protection Assay

The 811-bp *Pst*I fragment of IGF-II cDNA was inserted into pGEM 32. The plasmid was linearized with *Sal*I before transcription. A RNA probe was synthesized with a riboprobe kit according to the instructions of the manufacturer (Promega, Madison, WI) using the SP6 promoter. This resulted in a probe size of 300 bp containing 279 bp of IGF-II coding sequences and 21 bp of vector sequences. DNase-I (Promega) was added to digest the DNA template. Hybridization was carried out as previously described (34). Forty micrograms of total RNA were hybridized with 5×10^5 cpm probe in 30 μl buffer [80% formamide, 40 mM piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid), 0.4 M NaCl, and 1 mM EDTA] for 16 h at 45 C. Samples were then digested with 40 μg/ml RNase-A (Sigma) and 2 μg/ml RNase-T1 (Sigma) for 1 h at 30 C. The digestion was terminated with 20 μl 10% sodium dodecyl sulfate and 50 μg proteinase-K (Sigma) at 37 C for 15 min. The samples were then extracted with phenol-chloroform, precipitated with ethanol, and electrophoresed on a 5% polyacrylamide gel containing 8 M urea. The gel was dried and exposed to x-ray film for 16 h to 1 week. Authentic IGF-II mRNA will protect a 279-bp fragment from RNase digestion.

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Address requests for reprints to: C. Kent Osborne, M.D., Department of Medicine/Oncology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284–7884.

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† Deceased.

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