Progestins Induce Down-Regulation of Insulin-Like Growth Factor-I (IGF-I) Receptors in Human Breast Cancer Cells: Potential Autocrine Role of IGF-II

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Insulin-like growth factor-I (IGF-I) receptors are present in breast cancer cells and may play a role in breast cancer cell growth. We have studied the effect of progestins on IGF-I receptors in T47D human breast cancer cells. T47D cells constitutively express high levels of progesterone receptors and are a model for studying the regulation of cellular functions by progestins. Treatment of T47D cells with either progesterone or the synthetic progestin promegestone (R5020) decreased IGF-I receptor content by approximately 50%, as measured by Scatchard analysis and receptor biosynthesis studies. In contrast to progestins, estradiol, dexamethasone, and dihydrotestosterone did not influence IGF-I receptor content. No effect of R5020 was seen after 12 h of incubation, a near-maximal effect was seen after 24 h, and greatest effects were seen after 72 h. R5020 decreased IGF-I receptor mRNA abundance, indicating that progestins acted at the level of gene expression. However, progestins also increased the secretion of IGF-II, a ligand for the IGF-I receptor. In contrast to IGF-II, T47D cells did not express IGF-I. The addition of exogenous IGF-II to T47D cells down-regulated both IGF-I receptor binding and IGF-I receptor mRNA abundance. This study indicates, therefore, that progestins regulate IGF-I receptors in breast cancer cells and suggests that this regulation occurs via an autocrine pathway involving enhanced IGF-II secretion. (Molecular Endocrinology 5: 709–717, 1991)

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INTRODUCTION

Insulin-like growth factors (IGFs) are members of a family of peptide hormones that have a broad range of metabolic and mitogenic actions (1–3). The two principal members of this family are IGF-I and IGF-II. These growth factors have approximately 50% sequence homology and are also related to proinsulin (1, 2). In many tissues, the effects of IGF-I and IGF-II are mediated through the IGF-I receptor (2, 3). This receptor has structural and sequence homology to the insulin receptor and is a heterotetramer composed of two extracellular α-subunits (135 kDa) that bind the hormone and two transmembrane β-subunits (90–95 kDa) that have tyrosine kinase activity in their intracellular domains (2). Receptors for IGF-I have been reported in both human breast cancer specimens (4, 5) and cultured human breast cancer cell lines (4, 6–8). IGFs are potent mitogens when added to breast cancer lines in culture (4, 7, 8), and a specific monoclonal antibody to the IGF-I receptor blocks both the mitogenic effects of IGFs in cultured human breast cancer cells and the growth of breast cancer cells in nude mice (4, 7–10). Moreover, the IGFs are secreted by breast cancer cells and adjacent stromal tissues, suggesting that the IGFs act via autocrine and/or paracrine pathways (8, 11–15). Since these studies indicate that the IGFs and the IGF-I receptor may have a significant role in the biology of breast cancer cells, it is important to understand the potential mechanisms that regulate this ligand-receptor system.

Progestins have a variety of metabolic and proliferative effects on breast cancer cells (16, 17). Previously, it was reported that progestins increase insulin binding

to its receptor in human cultured breast cancer cells (18). Recently, we have observed that progestins increase insulin receptor gene expression and insulin receptor content in the T47D breast cancer cell line (19). In this cell line, progestins alone had no effect on cell growth, but markedly potentiated the mitogenic effect of insulin (19). The effect of progestins on IGF-I receptors, however, has not been previously investigated. In the present report we examined IGF-I receptor regulation by progestins in T47D cells. We found that progesterone down-regulates the IGF-I receptor at the level of gene expression. Moreover, progestins increase IGF-II secretion in T47D cells. These studies suggest, therefore, that progestins down-regulate the IGF-I receptor via an autocrine loop that involves increased secretion of IGF-II.

RESULTS

IGF-I Binding Specificity

We first characterized the IGF-I receptor in T47D cells by employing [^{125}I]IGF-I and unlabeled ligands in competition-inhibition studies (Fig. 1). T47D cells bound [^{125}I]IGF-I, and binding was competed for by unlabeled IGF-I at a half-maximal concentration (ED_{50}) of 0.8 nM. The ED_{50} for the related hormone IGF-II was approximately 9.0 nM. Monoclonal antibody αIR3, which specifically inhibits IGF-I binding to the IGF-I receptor (20), had an ED_{50} of 1.0 nM. In contrast, insulin, monoclonal antibody MA-10 (which specifically inhibits insulin binding to the insulin receptor) (21), and normal mouse immunoglobulin G (IgG) had little or no effect.

Influence of Progestins on [^{125}I]IGF-I Binding

We next tested T47D cells with various steroids: 17β-estradiol, dexamethasone, dihydrotestosterone, progesterone, and promegestone (R5020). Of the compounds studied, only progestins influenced the IGF-I-binding capacity (Fig. 2). Dihydrotestesterone, however, slightly lowered binding affinity. Both the synthetic progestin R5020 and progesterone, the latter at 1.0 μM, which is a physiological concentration (22), decreased IGF-I receptor-binding capacity by 30–40%. Further analysis of this progestin effect was carried out with R5020.

No effect of R5020 was seen after 12 h of incubation (Fig. 3), a near-maximal effect was seen after 24 h of incubation, and somewhat greater effects were seen after 72 h. The duration of incubation without R5020 had no consistent effect on IGF-I receptor-binding capacity. Scatchard analysis revealed that untreated T47D cells had a total IGF-I-binding capacity of 15,100 ± 1,780 sites/cell (mean ± SEM; n = 3), whereas 72 h after R5020 treatment, the binding capacity decreased to 7,840 ± 870 sites/cell. Since Scatchard plots were curvilinear, they were analyzed by a two-site model

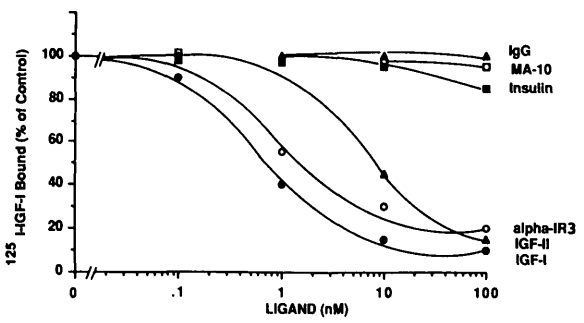
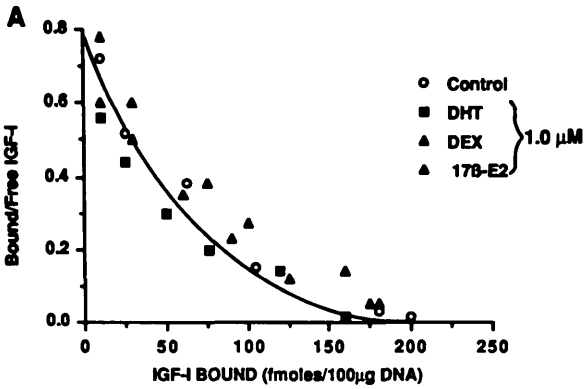


Fig. 1. Specificity of [^{125}I]IGF-I Binding to T47D Cells

Cells were incubated with [^{125}I]IGF-I and increasing concentrations of IGF-I, IGF-II, insulin, monoclonal antibodies αIR3 and MA-10, and normal mouse IgG. [^{125}I]IGF-I binding was carried out as described in *Materials and Methods*. Data are expressed as a percentage of the maximal [^{125}I]IGF-I bound. Each value is the mean of three separate experiments.



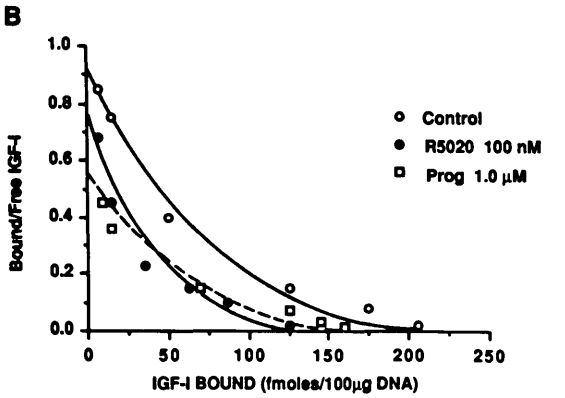


Fig. 2. Effects of Steroids on [^{125}I]IGF-I Binding to T47D Cells

Cells were plated in the absence of steroids, and 48 h later the medium was replaced with either control medium or medium containing the following steroids: 1 μM dihydrotestosterone (DHT), 1 μM dexamethasone (DEX), 1 μM 17β-estradiol (17βE_2), 100 nM R5020, and 1 μM progesterone. After 48 h of incubation, the cells were harvested, and [^{125}I]IGF-I binding was carried out. A representative of three Scatchard plots of the data are shown.

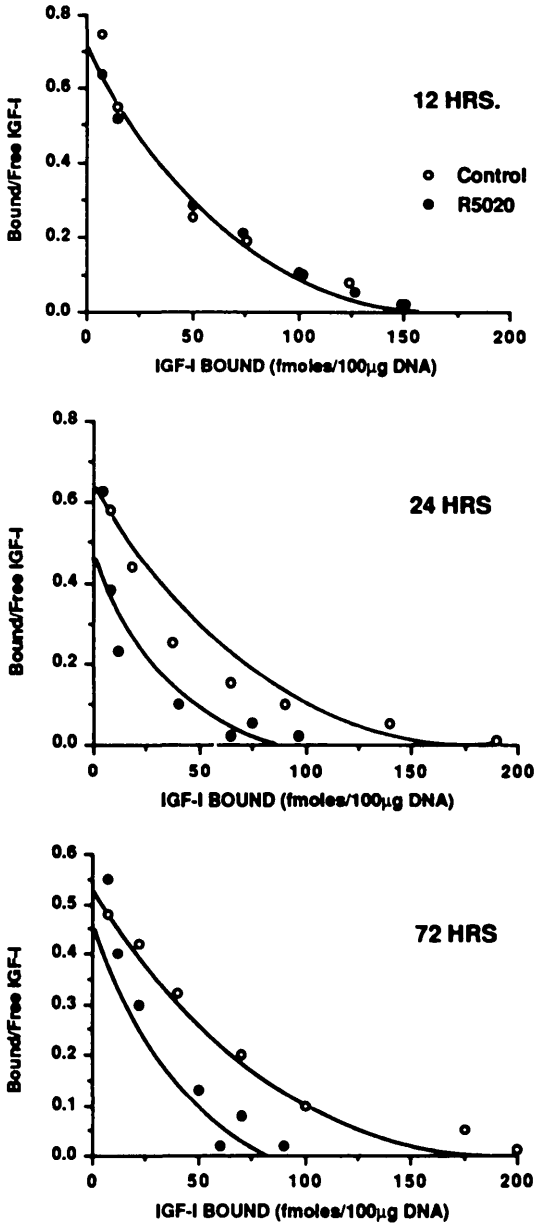


Fig. 3. Time Course of the Effect of R5020 on [^{125}I]IGF-I Binding to T47D Cells

Cells were plated in the absence of R5020, and 48 h later the medium was replaced with either control medium or medium containing 100 nM R5020. At the indicated times, cells were harvested and [^{125}I]IGF-I binding was carried out. Representative Scatchard plots of three experiments are shown.

(23). R5020 treatment (but not progesterone treatment) increased the affinity of the high affinity binding sites (from 1.8 ± 0.3 to 0.3 ± 0.1 nM; mean ± SEM; n = 3). R5020, however, did not alter the affinity of the lower affinity binding sites.

R5020 Effects on IGF-I Receptor Biosynthesis and mRNA Content

To determine whether R5020 had an effect on IGF-I receptor biosynthesis, T47D cells were preincubated for 48 h with 100 nM R5020, and then incubated with [^{35}S]methionine-cysteine in methionine-cysteine-free medium. Next, the cells were solubilized, and the receptors partially purified on wheat germ agglutinin agarose, immunoprecipitated with monoclonal antibody αIR3, reduced, and analyzed by polyacrylamide gel electrophoresis (Fig. 4). Pretreatment of T47D cells with R5020 was associated with a decrease in the synthesis of both the α- (135 kDa) and β-subunits (97 and 92 kDa) of the IGF-I receptor (Fig. 4). In T47D cells, as in other tissues, the IGF-I receptor β-subunit migrates as a doublet (2); the biosynthesis of both of these β-subunits was decreased.

To determine whether the decrease in IGF-I receptor biosynthesis was associated with a decrease in IGF-I receptor mRNA content, Northern blot analyses were carried out. Poly(A)$^+$ RNA was obtained (24) from cells that had been preincubated for 72 h in the presence and absence of 100 nM R5020. The poly(A)$^+$ RNA was normalized (24) and subjected to agarose gel electrophoresis, followed by transfer to nitrocellulose filters, and the filters were then probed with ^{32}P-labeled IGF-I receptor cDNA. In T47D cells, two major IGF-I receptor transcripts were detected on autoradiography at 11.0 and 7.0 kilobases (kb; Fig. 5). Treatment of T47D cells

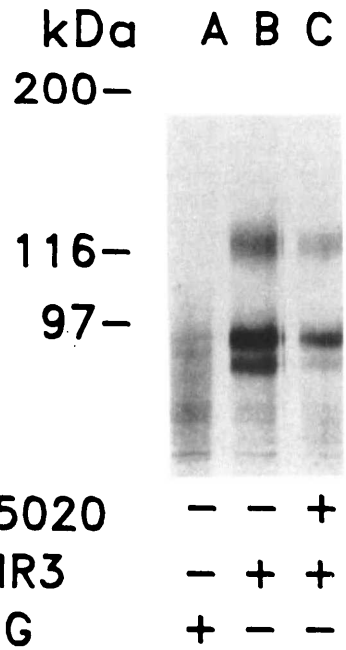


Fig. 4. Effect of R5020 on IGF-I Receptor Biosynthesis

T47D cells were preincubated for 48 h with 100 nM R5020. Cells were then labeled with 1 mCi [^{35}S]methionine-cysteine in medium lacking methionine and cysteine. After 16 h at 37 C, the cells were washed, collected by scraping, and solubilized in 1% Triton X-100 buffer. Equal amounts of wheat germ agglutinin-purified receptors were then immunoprecipitated with either monoclonal antibody αIR3 or normal mouse IgG. The immunoprecipitates were treated with 100 mM dithiothreitol, denaturated in Laemmli buffer, subjected to electrophoresis, and autoradiographed. A representative of three experiments is shown.

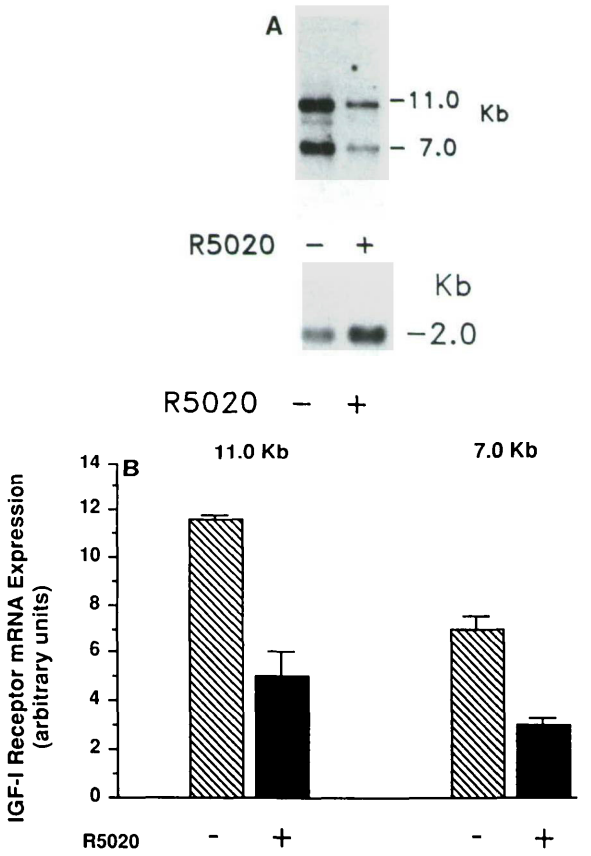


Fig. 5. Effect of R5020 on IGF-I Receptor mRNA

T47D cells were grown for 2 days without R5020 and then incubated for 3 days in the absence or presence of 100 nM R5020. Poly(A)$^+$ RNA was extracted and analyzed as described in *Materials and Methods*. A, Northern blot analysis of IGF-I receptor mRNA. The filter was first hybridized with labeled IGF-I receptor cDNA (*top*) and then washed and reprobed with labeled β-actin cDNA (*bottom*). A representative of three experiments is shown. A RNA ladder was used for the mol wt standards. Kb, Kilobases. B, Densitometry scanning of IGF-I receptor mRNA subunits. Values are the mean ± SEM of three experiments.

with 100 nM R5020 for 72 h resulted in an approximately 50% decrease in both mRNA species (Fig. 5). In contrast, and as previously reported (24), progestins slightly increased β-actin mRNA content.

Effect of R5020 on IGF-II Secretion

Progestin-induced down-regulation of IGF-I receptors could be explained by either a direct effect of steroid hormones on IGF-I receptor gene expression or an indirect mechanism, such as an autocrine loop involving IGF-I and/or IGF-II. To assess the latter possibility, we analyzed medium from T47D cells that were preincubated in the absence and presence of 100 nM R5020 for 72 h. To minimize the potential effects of IGF-binding proteins (25, 26), medium was subjected to extraction (see *Materials and Methods*) in order to dissociate the ligands from binding proteins. We then examined the ability of these extracts to inhibit the binding of [^{125}I] IGF-I to its receptor (Fig. 6). Extracts from R5020-treated cells were approximately 3-fold more potent in inhibiting [^{125}I]IGF-I binding than extracts from untreated cells (Fig. 6). Medium not exposed to cells was without effect. These data suggested, therefore, that T47D cells secreted significant amounts of either IGF-I or IGF-II (or both) in response to R5020.

We next used specific RIAs to directly measure IGF-I and IGF-II secretion. T47D cells produced detectable IGF-II immunoreactivity, and the amount of IGF-II released into the medium was approximately 10-fold greater after treatment with R5020 (Table 1). In contrast, no detectable IGF-I was seen in the medium of either control or treated cells (Table 1).

IGF-I and IGF-II mRNA Abundance

Since R5020 enhanced IGF-II secretion by T47D cells, we examined the effect of progestins on the gene expression of this growth factor. For this purpose poly(A)$^+$ RNA was obtained from both control and

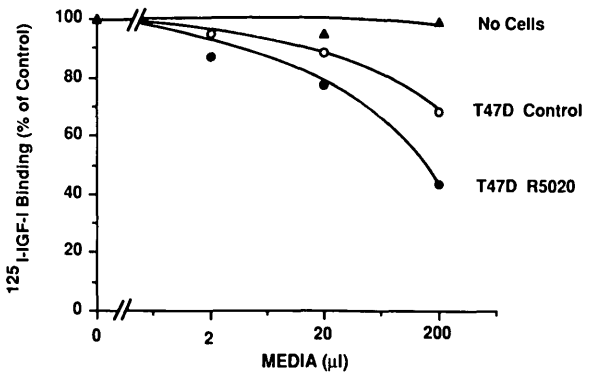


Fig. 6. Effect of Media from Control and R5020-Treated T47D Cells on [^{125}I]IGF-I Binding to T47D Cells

Cells were incubated in the presence or absence of 100 nM R5020 for 3 days. Media were collected, lyophilized, and acid-ethanol extracted. These extracts and extracts of media that were not exposed to cells were then tested for their ability to inhibit [^{125}I]IGF-I binding to T47D cells. A representative of three experiments is shown.

Table 1. Effect of Treatment with R5020 on IGF-I and IGF-II Secretion by T47D Cells

Treatment	IGF-I (nM)	IGF-II (nM)
Control	<0.8	3.6 ± 0.6
R5020 (100 nM)	<0.8	30.4 ± 2.8

Cells were plated in DMEM-H21 containing 5% FCS, glutamine, and antibiotics. At 50% confluency, the cells were washed with serum-free medium and incubated with serum-free medium with 1 μg/ml transferrin, 1 μg/ml fibronectin, and 25 mM HEPES with or without 100 nM R5020. After 24 h the medium was changed, and 3 days later the conditioned media were collected, lyophilized, and stored at −80 C until assayed. The amounts of IGF-I and IGF-II present were determined by specific RIAs (49, 50).

R5020-treated cells and subjected to Northern-blot analysis (Fig. 7). Under stringent hybridization and washing conditions, one IGF-II transcript of approximately 6.4 kb was detected in T47D cells; no change in IGF-II mRNA abundance was seen after R5020 treatment. In contrast, no specific mRNA bands for IGF-I were detected (data not shown).

In Vitro Down-Regulation of IGF-I Receptors by IGF-II

To directly assess whether IGF-II could down-regulate IGF-I receptors in T47D cells, we incubated them for 48 h in the presence of two different concentrations of IGF-II, 3 and 30 nM, corresponding to the IGF-II concentrations secreted in the absence and presence of R5020 (Table 1). After treatment with IGF-II, the bound hormone was removed from the cell surface by acid washing (27), and ligand binding assays were performed. IGF-II at 30 nM, but not at 3 nM, decreased the number, but not the affinity, of IGF-I receptors (Fig. 8). We next investigated whether down-regulation of IGF-I receptors by IGF-II occurred at the level of gene expression. Poly(A)$^+$ RNA obtained from control cells and cells exposed for 48 h to 30 nM IGF-II was subjected to Northern analysis (Fig. 9), and the filters probed with ^{32}P-labeled IGF-I receptor cDNA. After incubation with IGF-II, IGF-I receptor mRNA[1] content was decreased approximately 2-fold. The β-actin mRNA was not influenced by IGF-II.

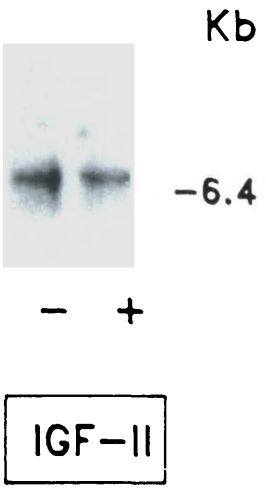


Fig. 7. Effects of R5020 on IGF-II mRNA
The Northern blot of poly(A)$^+$ RNA from Fig. 5 was washed and rehybridized with IGF-II cDNA. + and − refer to the presence and absence of 100 nM R5020. A representative of three experiments is shown.

[1] We noted that when T47D cells were incubated with serum, two major IGF-I receptor bands of approximately equal abundance were seen on Northern analysis (Fig. 5). In contrast, when cells were incubated under serum-free conditions, the larger band was more abundant. This observation raises the possibility that serum factors could regulate IGF-I receptor mRNA metabolism.

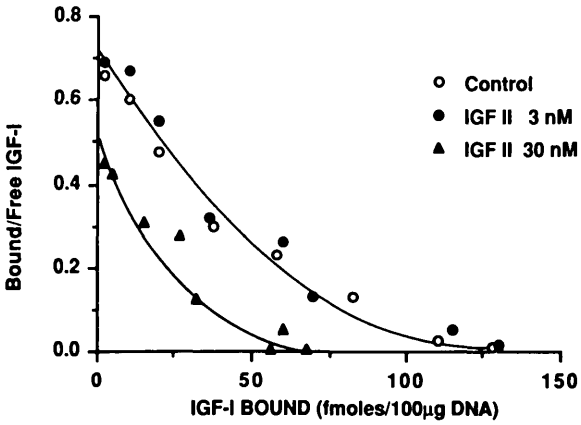


Fig. 8. Down-Regulation of IGF-I Receptors by IGF-II
Cells were grown in DMEM-H21 containing 5% FCS. At 50–60% confluency, the culture medium was replaced with serum-free DMEM containing either 1% BSA alone or 1% BSA plus 3 or 30 nM IGF-II. After 48 h of incubation, cells were acid washed to remove bound hormone and harvested, and [^{125}I] IGF-I binding was carried out. A representative of three Scatchard plots is shown.

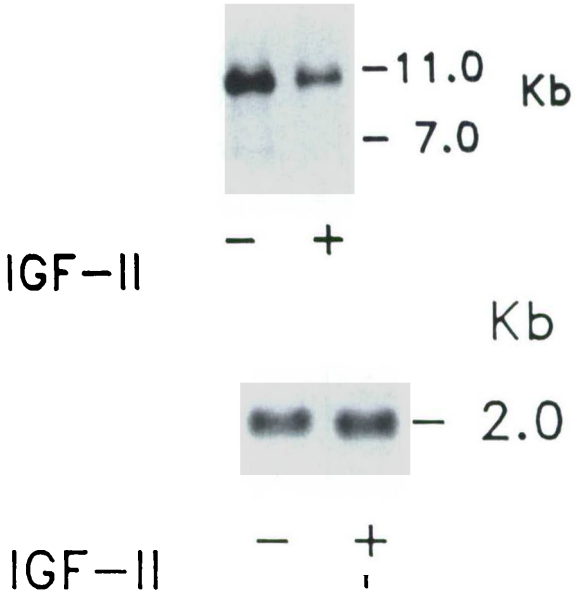


Fig. 9. Effect of IGF-II on IGF-I Receptor and β-Actin mRNA
T47D cells were grown for 3 days in DMEM-H21 with 5% FCS and then incubated for 48 h in serum-free medium containing 1% BSA in the presence or absence of 30 nM IGF-II. Poly(A)$^+$ RNA was extracted and analyzed. Filters were hybridized with labeled IGF-I receptor (*top*) and β-actin (*bottom*) cDNAs. A representative of three experiments is shown.

DISCUSSION

The growth of breast cancer cells is under the control of steroid and polypeptide hormones (15, 16, 28). In the present study we investigated progestin regulation of IGF-I receptors in the T47D human breast cancer cell line. Prior studies have documented that IGF-I receptors

are present in both normal breast tissue and breast cancer specimens (4, 5). In one study 93% of the breast cancer samples were positive for the presence of IGF-I receptors (5). Moreover, in several reports, IGF-I receptor expression was related to steroid receptor content (5, 29–32).

Expression of IGF-I and IGF-II, the major ligands for the IGF-I receptor, has been reported in breast cancer tissues (32–34). IGF-I transcripts were not detected in breast cancer cell lines, but IGF-I mRNA was found in all breast cancer specimens tested (33). In contrast, IGF-II transcripts were detected in both cultured breast cancer cells (8) and breast cancer specimens (34). These studies suggested, therefore, that IGF-I was produced by breast stromal cells, whereas IGF-II was produced by breast epithelial cells. These observations also raised the possibility that IGF autocrine and/or paracrine interactions may occur in breast cancer tissues (8).

In the present study with T47D cells, we analyzed the effect of progestins on both IGF-I receptors and IGF secretion. These cells were selected for two reasons. First, these cells have IGF-I receptors and secrete IGF or IGF-related proteins (4, 6–8, 34). Second, they constitutively express high levels of progesterone receptors without estrogen pretreatment, and thus, the effects of progestins on IGF-I receptor regulation can be studied independently of estrogen.

In the present study we found that treatment with either progesterone or the synthetic progestin R5020 down-regulated IGF-I receptors at the level of gene expression. Binding capacity, receptor biosynthesis, and IGF-I receptor mRNA levels were all decreased by approximately 50%. When the mechanism of this down-regulation was analyzed, it was found that the cells expressed IGF-II (but not IGF-I), and the secretion of this growth factor increased in response to progestins. The slow action of progestin on IGF-I receptor expression was consistent with an indirect effect on IGF-II.

The present study is the first to report that progestins increase IGF-II secretion. Voutilainen *et al.* (35) reported in ovarian granulosa cells that progesterone did not influence IGF-II mRNA content; however, in this study IGF-II secretion was not determined. In the present study progestins did not change IGF-II mRNA content, suggesting that steroid hormones acted to regulate the rate of IGF-II protein synthesis, protein degradation, or protein secretion[2]. Since the data suggested that the down-regulation of IGF-I receptors by progestins occurred via an autocrine pathway involving the increased secretion of IGF-II, we tested this hypothesis directly by demonstrating that exogenous IGF-II also down-regulated IGF-I receptor gene expression[3].

The ability of IGF-II to down-regulate the IGF-I receptor at the level of gene expression is not unique to breast cancer cells. We recently observed (36) that undifferentiated myoblasts demonstrated a high level of IGF-I receptor gene expression and low levels of IGF-II peptide gene expression. With differentiation, IGF-II secretion markedly increased, and IGF-I receptor mRNA abundance significantly decreased. These observations in muscle as well as in breast cancer cells suggest that IGF-I receptor expression in several tissues is regulated at least in part through autocrine secretion of IGF-II. Different mechanisms, however, may lead to increased IGF-II secretion. In muscle cells differentiation led to an increase in IGF-II mRNA abundance, whereas in breast cancer cells treated with progestins such an increase was not observed.

Other studies have shown that ligands can down-regulate their receptors at the level of gene expression. Low density lipoproteins down-regulate their receptors at the level of mRNA abundance (37). Moreover, insulin can also decrease the mRNA content of the insulin receptor (38).

A doublet appearance of the IGF-I receptor β-subunit, which has been described in several cell types (39–43), was seen in T47D cells, and both subunits were down-regulated by progestins. While differences in gel electrophoretic migration of the β-subunits have been ascribed to glycosylation differences in some cells (41), recent studies in the rat suggest that IGF-I receptor β-subunit heterogeneity may be accounted for by differences in peptide sequence, as determined by immunoprecipitation with domain-specific antireceptor antisera and tryptic phosphopeptide mapping (42, 43). Furthermore, an alternate mRNA transcript encoding the IGF-I receptor β-subunit has been recently identified (44). Phosphopeptide mapping studies also suggest that the two IGF-I receptor β-subunits differ from the insulin receptor β-subunit (42, 43).

In the present study R5020, but not progesterone, increased IGF-I receptor affinity. Previously, we reported that R5020 increased insulin receptor affinity (19). Also in the present study we observed that dihydrotestosterone slightly lowered IGF-I receptor affinity. The reasons for this steroid hormone regulation of peptide hormone receptor affinity are unknown, but may reflect a steroidal effect on other components of the plasma membrane, such as lipids, which are known to influence this function (45).

In summary, treatment of T47D human breast cancer cells with progestins is associated with decreased IGF-I receptor content, biosynthesis, and gene expression, with a concomitant increase in IGF-II peptide secretion. Further, treatment of T47D cells with IGF-II down-

[2] While progestins and other steroid hormones act primarily to increase gene transcription (55, 56), in some instances they have been shown to have other effects on cell function, such as RNA processing and turnover (55, 57, 58). The mechanism of the effect of progestins on IGF-II secretion, therefore, will necessitate further study.

[3] T47D cells also have IGF-II/mannose-6-phosphate receptors. It is possible, therefore, that IGF-II could down-regulate IGF-I receptors through a mechanism involving IGF-II/mannose-6-phosphate receptors.

regulated IGF-I receptor mRNA content. These studies suggest, therefore, that regulation of IGF-I receptor expression in breast cancer cells by progestins occurs through an autocrine mechanism involving enhanced secretion of IGF-II.

MATERIALS AND METHODS

Materials

The following were purchased: HEPES, bacitracin, phenylmethylsulfonylfluoride, hydroxymethylmethylglycine (Tricine), fibronectin, and all steroids from Sigma Chemical Co. (St. Louis, MO); BSA from Reheis (Chicago, IL); porcine insulin from Elanco Products Co. (Indianapolis, IN); ^{125}I-labeled IGF-I (2000 Ci/mmol) from Amersham (Arlington Heights, IL); [α-^{32}P] dCTP (3000 Ci/mmol), [^{35}S]protein labeling mix (methionine-cysteine; 1000 Ci/mmol), and promegestone (R5020) from New England Nuclear (Boston, MA); and protein-A-Sepharose from Pharmacia Laboratory Division (Uppsala, Sweden). All other reagents were of analytical grade.

Mouse monoclonal antibody MA-10 to the human insulin receptor was prepared as previously described (21). The following were gifts: mouse monoclonal antibody αIR3 to the IGF-I receptor (20) from Dr. S. Jacobs (Burroughs Wellcome Co., Research Triangle Park, NC), recombinant human IGF-I from Ciba Geigy (Summit, NJ), and recombinant human IGF-II from Eli Lilly (Indianapolis, IN). A 3.2-kb cDNA probe for the human IGF-I receptor was obtained as previously described (36). Complementary DNA for IGF-I (46) was a gift from M. Jansen. Complementary DNA for IGF-II (47) was purchased from American Type Culture Collection. The β-actin cDNA (48) was a gift from P. Gunning.

Cell Culture

T47D cells (from Drs. C. Sonnenschein and A. Soto, Tufts University, Boston, MA) were routinely grown in Dulbecco's Modified Eagle's medium (DMEM)-H21 containing 5% fetal calf serum (FCS), nonessential amino acids, glutamine, transferrin, penicillin, streptomycin, and biotin (Cell Culture Facility, University of California, San Francisco). Steroids were dissolved in ethanol and added directly to the medium. Control cells received ethanol only.

Collection of Media for RIA of Secreted IGF-I and IGF-II

Cells were plated in T 150-cm^2 flasks in DMEM-H21 containing 5% FCS and grown to 50% confluency. To measure IGF-I and IGF-II secretion, cells were washed with serum-free medium, and the medium was changed to serum-free DMEM with 25 mM HEPES, pH 7.4, and 1 μg/ml each of transferrin and fibronectin. R5020 (100 nM) was added to appropriate cells. After 24 h, medium was changed, and the first collection was discarded. Three days later, the medium was collected and centrifuged to remove cellular debris, and aprotinin was added to yield 0.23 trypsin inhibitor units/ml. The samples were then lyophilized, redissolved in water, and stored at −80 C until assayed.

RIAs for IGF-I and IGF-II were carried out by Dr. Mark Stene, Endocrine Sciences (Tarzana, CA). For IGF-I determination, binding proteins were removed by G-50 chromatography in 0.25 M formic acid, as described by Hintz *et al.* (49). For IGF-II, binding proteins were removed by extraction with 0.8 M formic acid, 0.05% Tween-20, and 70% acetone, according to the method of Bowsher *et al.* (50). Antibody UBK 487 was used in the IGF-I RIA (51); the assay sensitivity was 12 ng/ml. For IGF-II determinations, a monoclonal antibody against human IGF-II (Amano Pharmaceutical Co., Nagoya, Japan) was used; the assay sensitivity was 10 ng/ml.

[^{125}I]IGF-I Binding Assay for Measurement of IGF-I Receptors

T47D cells were studied when they were in the late log phase of growth. Monolayers were harvested by a 10-min incubation at 37 C with 1 mM EDTA in PBS. The cells were then resuspended at 2–3 × 10^6 cells/ml in binding buffer (DMEM containing 1% BSA, 25 mM Tricine, and 25 mM HEPES, pH 7.6). Binding studies were carried out in triplicate in 500 μl binding buffer containing [^{125}I]IGF (12 pM) with or without unlabeled IGF-I (10 pM-100 nM). After incubation for 3 h at 16 C under shaking, the cells were washed twice at 4 C with 10 mM Tris-154 mM sodium chloride, pH 7.5, then dissolved in 0.3 mg/ml sodium dodecyl sulfate, and the radioactivity was counted. Total binding of [^{125}I]IGF-I to these cells was in the range of 25%/100 μg DNA. Nonspecific binding (<1% of the total radioactivity) was subtracted from total binding, and data were normalized for DNA content (52). Competitive binding studies were analyzed by nonlinear least square computer program to obtain receptor affinity and capacity and then transformed according to the method of Scatchard (23).

The ability of extracts of conditioned medium from T47D cells to inhibit [^{125}I]IGF-I binding to its receptor in these cells was also examined. Media were collected as they were for RIA, and IGF-binding proteins removed, as previously described (26). Samples were then reconstituted in binding buffer and incubated as described above for binding studies.

IGF-I Receptor Down-Regulation Studies

Down-regulation experiments were carried out by replacing the culture medium with serum-free medium containing 1% BSA with or without two concentrations of IGF-II. After 48 h of incubation, cells were washed twice at 4 C with 150 mM NaCl (pH 4.5) containing 0.5 M Na acetate for 5 min to remove the ligand from the cell surface (27). The cells were then washed twice with PBS and harvested. Ligand binding assays were performed as described above.

RNA Extraction and Northern Blot Analysis

Poly(A)$^+$ RNA was prepared from T47D cells using a one-step proteinase-K method and oligo(dT)-cellulose chromatography (24). RNA was normalized as previously described, employing optical density and then probing with oligo(dT) (24). Seven micrograms of poly(A)$^+$ RNA were denaturated, electrophoresed in 1–1.5% agarose gel containing 2.2 M formaldehyde, and then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) (53). Complementary DNAs for IGF-I receptor, IGF-I, IGF-II, and β-actin were labeled with ^{32}P to specific activities of 0.5–1.0 × 10^9 cpm/μg. Hybridization was carried out for 48 h at 42 C, as previously described (24). After hybridization, the final wash was in 0.1 × SSC (1 × SSC = 150 mM NaCl and 15 mM citric acid, pH 7.0)-0.1% sodium dodecyl sulfate at 55 C.

Metabolic Labeling and Immunoprecipitation of IGF-I Receptor

T47D cells were plated in 75-cm^2 flasks and grown in the presence or absence of 100 nM R5020 for 2 days. The flasks were washed twice with methionine- and cysteine-free DMEM-H21 with 5% dialyzed FCS and 2 mM glutamine, and then incubated for 16 h at 37 C with 1 mCi [^{35}S]methionine-cysteine. Equal numbers of cells were washed twice with PBS, scraped from the flasks, and solubilized for 60 min at 4 C in 50 mM HEPES buffer (pH 7.6) containing 1% Triton X-100 plus protease inhibitors (2 mg/ml bacitracin, 0.1 mM phenylmethylsulfonylfluoride, and 0.1 mM leupeptin). After solubilization, the

suspensions were centrifuged at 100,000 × *g* for 60 min at 4 C. The supernatant solutions were purified through wheat germ agglutinin columns and normalized for glycoprotein radioactivity (54), and the semipurified receptors were incubated with 3 μg/ml of either monoclonal antibody αIR3 or normal mouse IgG at 4 C for 16 h. The antigen-antibody complex was then precipitated by the addition of protein-A-Sepharose that had been precoated with rabbit antimouse IgG. The protein-A pellets were washed, denaturated in Laemmli buffer with 100 mM dithiothreitol, and subjected to 7.5% polyacrylamide gel electrophoresis and autoradiography.

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