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Progestin regulation of insulin and insulin-like growth factor I receptors in cultured human breast cancer cells

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Summary

Recent studies indicate that the insulin receptor (IR) content is higher in breast cancer cells than in normal mammary epithelial cells. This observation has been made both in tissue specimens from patients with breast cancer, and in various human cultured breast cancer cell lines. Investigations have now been undertaken to understand the role of progestins in the regulation of the IR and the closely related insulin like growth factors-I receptor (IGF-I-R). Pretreatment of T-47D cultured human breast cancer cell lines with progestins induced a time and dose dependent increase in IR content. This increase was due primarily to an effect of progestins to increase IR mRNA levels. Other steroid hormones including glucocortocoids, estrogen, and testosterone were without effect. In contrast to their up-regulation of the IR, progestins down-regulated the IGF-I-R at the level of mRNA. An analysis of the processes involved revealed that progestins increased the biosynthesis of a ligand for IGF-I receptor, IGF-II. IGF-II in turn down-regulated the IGF-I-R. Thus these studies indicate that progestins have important effects on both the IR and the IGF-I-R. The effects of progestins on these and other growth factor receptors, therefore, may have an important role in the biology of breast cancers.

Introduction

Human and animal breast carcinomas have receptors for steroid and peptide hormones, and both in vitro and in vivo studies have indicated that tumor proliferation rate and overall growth are hormone-dependent [1,2]. Approximately one-third to one-half of all breast cancer cases show estrogen-dependent growth, and clinical studies have indicated that patients whose tumors have high levels of estrogen and progestrerone receptors (ER, PR) have biologically less aggressive tumors and have a better prognosis than patients with ER and/or PR negative tumors [3,4]. However, some receptor-positive breast cancers are not responsive to endocrine treatment involving steroid hormones, behave much more aggressively, and reduce patient survival [4,5]. One possibility is that the more aggressive tumor cell behavior is derived from the influence of other hormones

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such as polypeptide hormones and growth factors. These latter factors may play an autocrine and/or a paracrine role in determining aggressive breast tumor behavior, a more rapid tumor proliferation rate, and steroid hormone treatment unresponsiveness.

Recent investigations have focused on growth factors and receptors that are members of the tyrosine kinase family [6]. A number of growth factors stimulate cellular mitogenesis by interacting with a family of cell surface receptors that possess an intrinsic ligand-sensitive protein tyrosine kinase activity that intitiates changes in both cell metabolism and growth. Tyrosine kinase receptors are typically composed of an extracellular ligand binding domain linked to a cytoplasmically-oriented catalytic domain, that not only transduces the growth factor or hormonal signal, but also generates mitogenic second messengers. At present, four subclasses of tyrosine kinase receptors have been identified [6]. Subclass II receptors include the IR and the closely related IGF-I-R which are activated by insulin and by IGF-I and IGF-II, respectively. IGFs are members of a family of peptide hormones that have a broad range of metabolic and mitogenic actions [7-9]. The two principal members of this family are IGF-I and IGF-II. These two growth factors have approximately 50% sequence homology, and are also related to proinsulin [7,8]. In many tissues, the effects of IGF-I and IGF-II are mediated through the IGF-I-R [8,9]. The IGF-I-R has structural and sequence homology to the IR. Both the IR and the IGF-I-R are heterotetramers 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 [10]. The IR and IGF-I-R have 50% sequence homology and both of them, when activated by their respective ligands, stimulate cell growth. Moreover, overexpression of both receptors into nontransformed cells leads to a ligand-induced transformed phenotype. How these receptors are regulated in breast cancer cells and whether steroid hormones affect their expression and function are still not completely understood. This information is of general interest since sex steroid hormone administration is a frequent clinical occurence in women, and estradiol is a well known growth promoting hormone in breast cancer cells.

The effects of progesterone on the growth of breast cancer cells have been undefined. Both in vivo and in vitro studies have indicated that progesterone antagonizes estrogen-stimulated growth of endometrial cancer cells and normal uterine epithelial cells. This effect occurs by down-regulation of estrogen receptors [11-13]. However, when similar studies were carried out with breast cancer cells, progesterone or synthetic progestins either inhibited [14,15], stimulated [16], or had no effect [17] on cell growth. One explanation for these conflicting results in breast cancer cells is that there are interactions between progesterone and growth-promoting hormones which have not yet been elucidated. The present studies were undertaken to investigate progestin growth factor receptor interactions.

Insulin receptor - progestin interactions

Insulin is a known growth-promoting hormone for breast tissues. In cell culture insulin stimulates the growth of both human breast cancer cells [18] and normal human breast epithelial cells [19]. Moreover, several human breast cancer cell lines in permanent culture have specific, high-affinity receptors for insulin [18], and the presence of IR has been reported in frozen specimens of human breast carcinomas [20,21].

We have recently developed a new, sensitive, and specific radioimmunoassay for the direct measurement of IR [22]. This assay was then employed to measure IR content in surgical specimens of breast cancer [21]. The IR content of 159 specimens averaged 6.15 $\pm$ 3.69 ng/0.1 mg

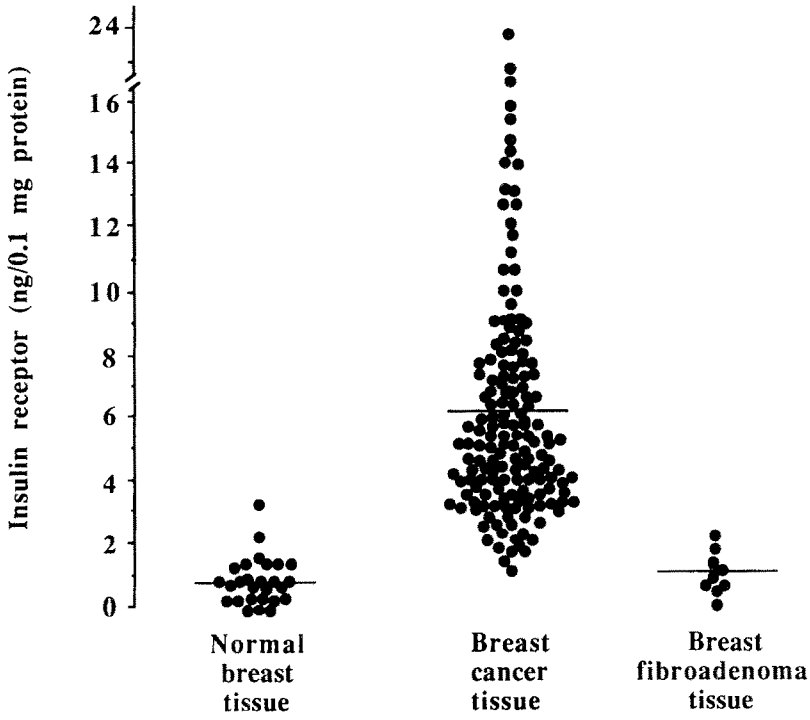

Figure 1. Insulin receptor content measured by radioimmunoassay in normal breast tissues, breast cancer tissues, and fibroadenomas. From reference 21 with permission.

protein (Figure 1). This value was more than sixfold higher than the mean value found in normal breast tissues obtained at total mastectomy, and in normal specimens obtained from reduction mammoplasty. Immunostaining of the specimens revealed that the IR content was high in malignant epithelial cells but not in stromal and inflammatory cells [21]. Statistical analysis revealed that the IR content of the tumors correlated positively with tumor size, and histological grading suggesting that IR may play a role in the biology of these tumors [21].

Whether progesterone interacts with insulin in regulating breast cancer cell growth has not been explored. In order to investigate the effect of progestins on the IR we used T47D human breast cancer cells [23]. These cells were chosen for the majority of these studies for two reasons. First, it was previously reported that progestin treatment increases IR binding in this cell line [15]. Second, these cells express high basal levels of progesterone receptors [24] and, therefore, estrogen treatment is not needed for progesterone receptor gene expression as is the case with other normal and malignant breast cell lines.

Treatment of T47D cells with the synthetic progestin R5020 induced a time and dose-dependent increase in IR content, as measured by both ligand binding studies (Figure 2a,b) and radioimmunoassy (not shown). Progesterone was 10-fold less potent than R5020; cortisol, 17β-estradiol, and dihydrotestosterone had little or no effect (Figure 2b). In these studies the IR monoclonal antibody MA-10, which inhibits insulin binding to the IR but not the IGF-I-R, inhibited insulin binding to T47D cells (not shown), indicating that ^{125}I-insulin was bound to its specific receptor and not to the IGF-I-R. Progestin treatment increased both IR mRNA levels and also the relative distribution of multiple

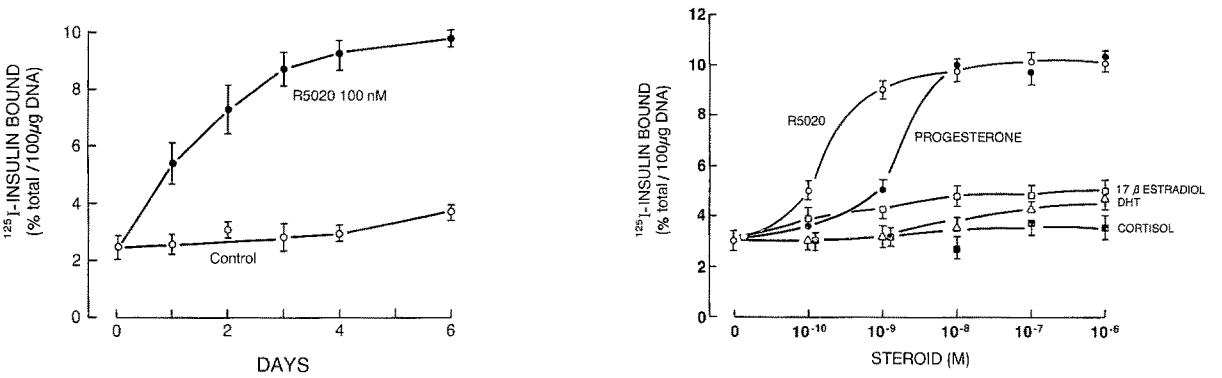

Figure 2. A) Time course of R5020 stimulation of ^{125}I-insulin binding to T47D cells. Cells were plated in the absence of R5020, and 48 h later (day 0) the medium was replaced with either control medium or medium containing 100 nM R5020. At the indicated times, cells were harvested and ^{125}I-insulin binding was carried out. B) Influence of steroid hormones on ^{125}I-insulin binding to T47D cells. As in experiments shown in A, at 48 h the medium was replaced with medium containing the various steroid hormones at the concentrations shown. After 4 days of treatment, cells were harvested and specific ^{125}I-insulin binding was measured. DHT= dihydrotestosterone. From reference 23 with permission.

IR mRNA transcripts (Figure 3). Insulin alone only had a small effect on cell growth, and progesterone by itself (in the absence of estrogens) was without effect (Figure 4). However, when cells were pretreated with progesterone, there was a marked potentiation of insulin-stimulation of cell growth (Figure 4). Similar effects were seen in MCF-7, an estrogen sensitive breast cancer cell line [18] (Figure 5). These studies in breast cancer cells demonstrated, therefore, that IR gene expression is under the regulation of progestins, and raised the possibility that progestin-insulin interactions may regulate breast cancer cell growth in vivo.

IGF-I receptor - progestin interactions

The presence of IGF-I-R has been reported in both human breast cancer specimens [25,26] and in cultured breast cancer cells [27-33]. IGF-I and IGF-II are potent mitogens for cultured breast cancer cells [27-33], and their effects are mediated through the IGF-I-R. In fact, a monoclonal antibody to the IGF-I-R has been reported to block the growth of breast cancer cells both in

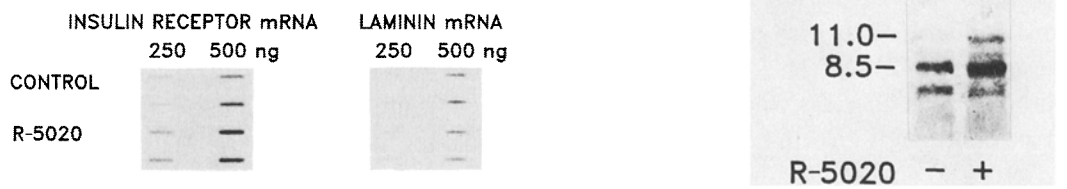

Figure 3. Effect of R5020 on insulin receptor mRNA. T47D cells were grown for 2 days without R5020 and then incubated 4 days in the absence or presence of 100 nM R5020. A) slot blot of poly(A)+ RNA from control and R5020-treated cells. Filters were probed with either labeled insulin receptor cDNA or labeled lamin cDNA. B) Northern blot of insulin receptor poly(A)+ RNA from control (–) and R5020-treated (+) cells. A representative of 4 different experiments is shown. An RNA ladder was used as the molecular weight standard. Kb=kilobases. From reference 23 with permission.

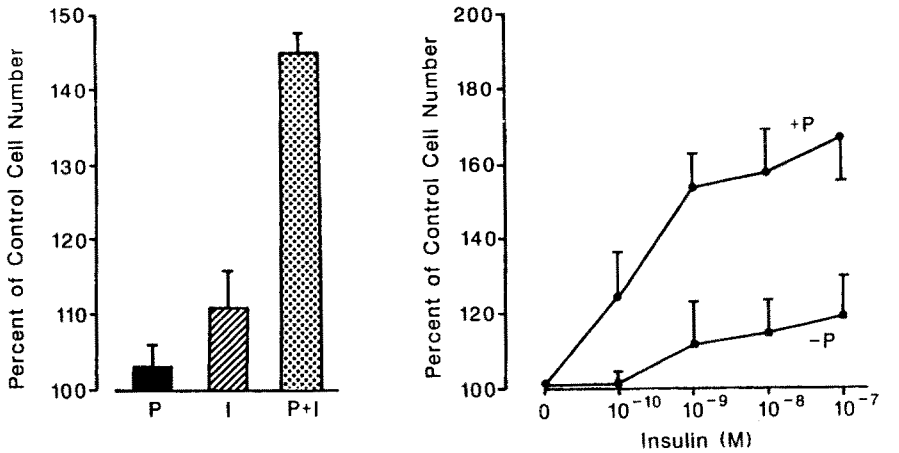

Figure 4. Effect of progesterone on the growth response of T47D cells to insulin. On the left, after 1 day of culture cells were continuously incubated in the presence or absence of 30 nM progesterone for the entire experiment. After 2 days of treatment, 10 nM insulin was added for 6 days. P = cells treated with progesterone; I = cells treated with insulin; P+I = cells treated with progesterone and insulin. On the right, cells were preincubated for 48 h in either the presence (+P) or absence (–P) of 30 nM progesterone and then treated for 6 days with different doses of insulin. Cells were harvested and cell nuclei counted as described. From reference 23 with permission.

vitro and in vivo [30,31]. Both IGF-I and IGF-II are secreted by breast cancer epithelial cells and

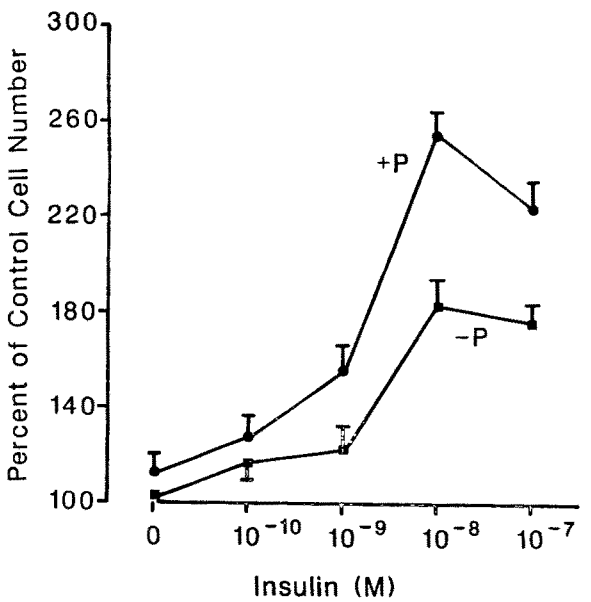

Figure 5. Effect of progesterone on the growth response of MCF7 cells to insulin. MCF7 cells were preincubated in the presence (+P) or absence (–P) of 30 nM progesterone and then treated with insulin as described for T47D cells. From reference 23 with permission.

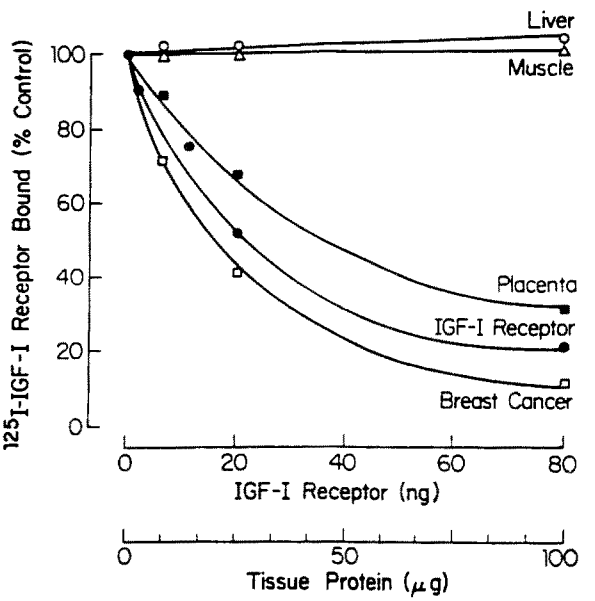

Figure 6. Competition-inhibition curves of various tissue extracts on the binding of ^{125}I-IGF-I receptor to anti-IGF-I receptor antiserum. Liver, muscle, placenta, and breast cancer tissues were processed and measured in the specific IGF-I-R radioimmunoprecipitation assay as previously described. The control competition inhibitor curve using a highly purified IGF-I-R standard is also shown (●). From reference 34 with permission.

adjacent stromal cells, suggesting potential autocrine-paracrine regulation in breast cancer [27-33].

By using a specific radioimmunoassay for the IGF-I-R [34] we observed that both breast cancer tissues from patients and cultured breast cancer cells showed detectable IGF-I-R contents, which correlated with IGF-I ligand binding (Figure 6).

Interactions between IGF-I-R and steroid hormones have not been extensively explored.

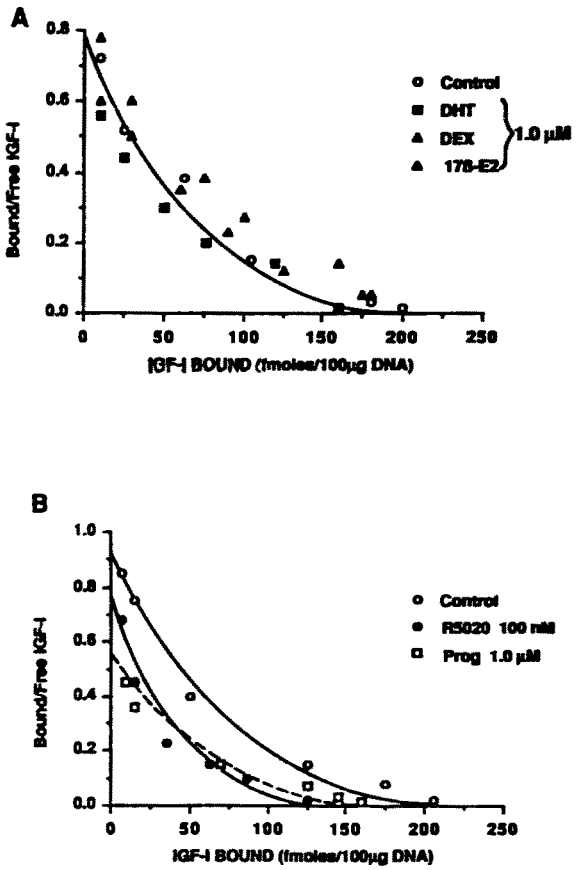

Figure 7. Effects of steroids on ^{125}-IGF-I binding to T47D cells. Cells were plated in the absence of steroids, and 48h later the medium was replaced with either control medium or medium containing the following steroids: 1 µM dihydrotestosterone (DHT), 1 µM dexamethasone (DEX), 1 µM 17β-estradiol (17βE2), 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. From reference 36 with permission.

Estradiol increases IGF-I binding and IGF-I-R mRNA content in MCF-7 cells [35]. We then

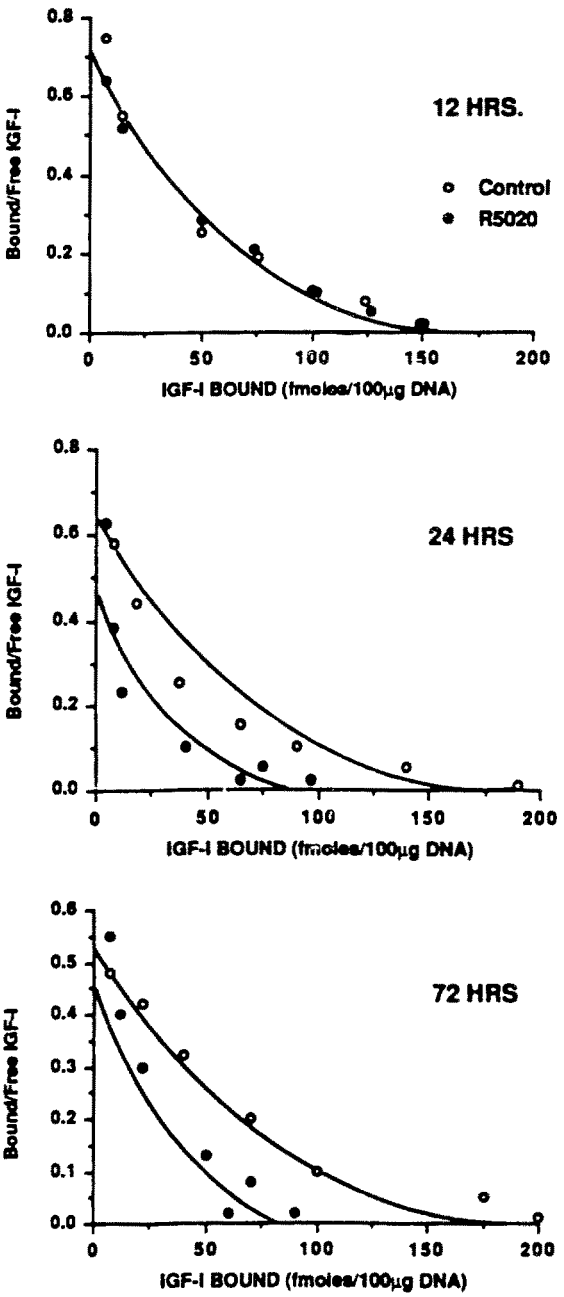

Figure 8. Time course of the effect of R5020 on ^{125}I-IGF-I binding to T47D cells. Cells were plated in the absence of R5020, and 48 h later the medium was replaced with either control medium or medium containing 100 nM R5020. At the indicated times cells were harvested and ^{125}I-IGF-I binding was carried out. From reference 36 with permission.

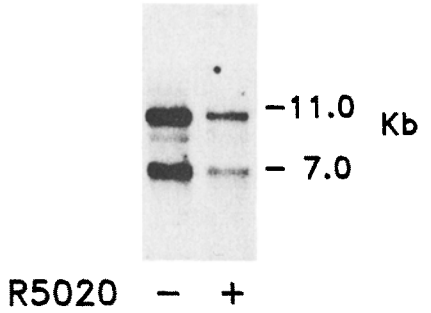

Figure 9. Effect of R5020 on IGF-I-R mRNA. T47D cells were grown for 2 days without R5020 and then incubated for 3 days in the absence or presence of 100 nM R5020. Poly(A)+ RNA was extracted and analyzed by Northern blot. A representative of three experiments is shown. An RNA ladder was used for the mol wt standards. Kb = kilobases. From reference 36 with permission.

investigated the effects of progestins [36]. Treatment of T47D cells with either progesterone or the synthetic progestin promegestone (R5020) decreased IGF-I-R content by approximately 50%, as measured by Scatchard analysis and receptor biosynthesis studies (Figure 7). R5020 was 10 fold more potent than progesterone. In contrast to progestins, estradiol, dexamethasone, and dihydrotestosterone had no influence on IGF-I-R content. No effect of R5020 was seen after 24 h, and greatest effects were seen after 72 h (Figure 8).

Table 1. Effect of treatment with R5020 on IGFI 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 medium was collected, lyophilized, and stored at -80° C until assayed. The amounts of IGF-I and IGF-II present were determined by specific radioimmunoassays. From reference 36 with permission.

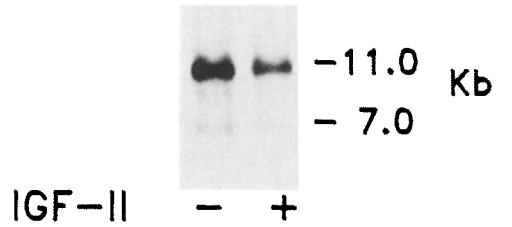

Figure 10. Effect of IGF-II on IGF-I receptor mRNA. T47D cells were grown for 3 days in regular DMEM and then incubated for 47 h in serum-free medium containing 1% BSA in presence or absence of 40 nM IGF-II. Poly(A)+ RNA was then extracted and analyzed. Filters were hybridized with labelled IGF-II receptor cDNA. From reference 36 with permission.

In T47D cells, R5020 decreased IGF-I-R mRNA abundance (Figure 9), indicating that progestins acted at the level of gene expression.

Other studies indicated that progestins increased the secretion of IGF-II in T47D cells (Table 1). T47D cells did not express IGF-I. When exogenous IGF-II was added to T47D cells, there was a down-regulation both of IGF-I-R binding and IGF-I-R mRNA abundance (Figure 10). The present studies indicate, therefore, that progestins regulate IGF-I-R in breast cancer cells, and suggest that this regulation occurs via an autocrine pathway involving enhanced IGF-II production.

Discussion

Our studies demonstrate that progestins regulate both the IR and IGF-I-R in breast cancer cells. Since these receptors are structurally similar, it was expected that progestin would act similarly on both proteins. However, progestins up-regulated the IR and down-regulated IGF-I-R. Moreover this activation occured via different mechanisms.

In most cells that have been studied, several IR mRNA species are observed in agarose gels with molecular sizes ranging widely from 5.2 to 11 kilobases [10]. Studies of these multiple

transcripts suggest that they are due to variable splicing at the 3' end of the IR RNA [10,37]. There are multiple 5' start sites but they differ only by several hundred bases [38], and thus do not account for the size heterogeneity. While these multiple species are all involved in IR protein synthesis (since they are all found associated with ribosomes) [39], the biological role for these multiple mRNA species is unknown. In T47D cells we observed that progestin treatment increased the relative content of the higher molecular weight mRNA forms. While progestins and other steroid hormones act primarily to increase gene transcription [39,40], in some instances they have been reported to alter RNA processing and turnover [40-42]. The present study of the IR in T47D cells provides a new example, therefore, of steroid hormone regulation of mRNA metabolism.

In the present study we observed that the increment in IR mRNA content was paralleled by an increase in IR protein content as measured by both Scatchard analysis and radioimmunoassay. In addition to changing binding capacity, progestin treatment also altered the affinity of the high affinity insulin-binding sites. The reason for this effect is unknown, but it may reflect an action of progestins on other elements of the plasma membrane such as lipids which, in turn, are known to influence receptor affinity [10].

Progestins have been reported to increase insulin binding to its receptor in ZR75 human breast cancer cells [43] and in a variant subline of T47D [26], but the mechanism of this up-regulation was not investigated. We have now observed that progesterone pretreatment of both T47D cells and MCF7 cells enhances insulin stimulation of cell growth. This observation indicates that up-regulation of IR by progestin in human breast cancer cells is directly related to enhanced insulin action.

In the present study we also found that progestin treatment down-regulated the IGF-I-R, and that this effect occurred at the level of gene expression. Binding capacity, receptor biosynthesis, and IGF-I-R 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 that the secretion of this growth factor increased in response to progestins. The slow action of progestins on IGF-I-R expression was consistent with an indirect effect via IGF-II.

The present study is the first to report that progestins increase IGF-II secretion. In ovarian granulosa cells it has been reported that progesterone did not influence IGF-II mRNA content [44]; however, in this study IGF-II secretion was not determined. Since the present data suggested that the down-regulation of IGF-I-R 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-R gene expression.

The ability of IGF-II to down-regulate the IGF-I-R at the level of gene expression is not unique to breast cancer cells. We have recently observed [45] that undifferentiated myoblasts demonstrated a high level of IGF-I-R gene expression and low levels of IGF-II peptide gene expression. The observations in muscle as well as in breast cancer cells suggest that IGF-I-R expression in several tissues is regulated at least in part through an autocrine effect involving IGF-II.

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 [46]. Moreover, insulin can also decrease the mRNA content of the IR [47]. In conclusion, taken together our studies suggest that IGF-I-R expression in breast cancer cells is regulated by progestins through an autocrine mechanism involving enhanced secretion of IGF-II.

The present observations in cultured human breast carcinoma cells demonstrating an inter-

action between growth factor receptors and steroid hormones may have clinical implications. We have recently observed that both IR and IGF-I-R are elevated in breast carcinomas when compared with normal breast tissues. It is possible, therefore, that the interaction between progesterone, insulin, IGF-I, and their receptors in breast cancer cells may also occur in vivo and that this interaction may have a role in regulating the growth of breast tumors. These findings may also be relevant for a better understanding of the mechanisms controlling the proliferation rate of normal breast epithelial cells. In the presence of insulin, progesterone stimulates the growth of cultured mammary epithelial cells in rodents [48]. Also, several studies have shown that the mitotic activity of normal breast epithelial cells is highest during the luteal phase of the menstrual cycle when progesterone levels are highest [49]. Moreover, a slightly higher risk for breast cancer has been reported in women using oral contraceptives containing estrogen and progesterone. Therefore, further studies of steroid hormone growth factor interactions are needed to define the roles of these hormones and factors and their receptors in breast cancer biology.

Acknowledgements

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