Receptor Binding and Growth-promoting Activity of Insulin-like Growth Factors in Human Breast Cancer Cells (T-47D) in Culture

Yvonne Myal, Robert P. C. Shiu, Banani Bhaumick, and Marvin Bala

ABSTRACT

Insulin-like growth factors (IGFs) and insulin are known to be mitogenic to a variety of cell types, although a growth-regulatory role of IGFs on human breast cancer cells has not yet been fully investigated. In the present study, we examined the receptor binding and the effect on growth of IGFs and insulin in a human breast cancer cell line (T-47D). Specific binding of 125I-basic somatomedin (BSM/IGF-I), 125I-multiplication-stimulating activity (MSA III-2/IGF-II), and 125I-insulin has been demonstrated in monolayer T-47D cells grown on plastic substratum. When the binding of 125I-BSM and 125I-MSA III-2 was studied, unlabeled BSM and unlabeled MSA were the most effective competitors for the respective binding sites. Unlabeled insulin at high concentration also inhibited the binding of 125I-BSM and 125I-II. For 125I-insulin binding, however, unlabeled MSA III-2 and MSA II were more effective than unlabeled insulin in displacing 125I-insulin from its binding sites. These observations suggest that the binding sites for IGF-I and IGF-II are distinct in T-47D cells and that insulin cross-reacts weakly with IGF-I and IGF-II binding sites. BSM (IGF-I) and MSA (IGF-II) (1 µg/ml) produced a 1.5-fold increase in cell proliferation of T-47D cells grown on plastic substratum. The mitogenic effect of IGFs on T-47D was more apparent when cells were grown on collagen gel. At 500 ng/ml, MSA III-2, BSM, and insulin stimulated cell growth 4-, 2.5-, and 1.5-fold, respectively. Our results suggest that the IGFs may be involved in the growth regulation of human breast cancer cells.

INTRODUCTION

The IGFs (somatomedins) refer to a family of closely related mitogenic polypeptides. These peptides can be placed into 2 groups based on their isoelectric points. The basic group, with >7.5 isoelectric points, include IGF-I, SM-C, and BSM. Recently, it has been established that IGF-I is identical with SM-C (14). The BSM (1) is believed to be very similar, if not identical, to IGF-I and SM-C (2). The acidic-neutral group, with <7.5 isoelectric points includes IGF-II and MSA. MSA (rat IGF-II) was shown to be homologous with the human counterpart IGF-II (16). MSA was first isolated from the serum-free culture medium harvested from the Buffalo rat liver cell line, BRL 3A (5) and consists of a family of biologically active polypeptides, 2 of these species (MSA II-1 and MSA II-2) were purified to homogeneity (19). The secretion of circulating levels of the basic group (IGF-I/SM-C/BSM) is dependent on pituitary growth hormone and has been suggested to be an adult growth factor (31). The secretion of the acidic-neutral somatomedins are only partially dependent on pituitary growth hormone (31). However, because of their high concentration in fetal blood, they have been implicated to be important fetal growth factors (4, 20).

The IGFs share common biological activities with insulin, presumably due to their structural similarity (27). It has already been shown that insulin can influence the growth of human mammary gland in organ cultures (3) and some human breast cancer cells in long-term culture (22, 23). In general, however, the mitogenic activity of insulin in many cell types could only be observed with supraphysiological concentrations of insulin (10, 24, 29). This led to the speculation that perhaps the weak mitogenic effect of insulin is primarily due to its structural similarity to the IGFs. The latter peptides may be more likely candidates as physiological growth regulators of breast cancer cells. To evaluate this possibility, we decided to compare the mitogenic effect and receptor binding of IGFs and insulin in human breast cancer cells.

MATERIALS AND METHODS

Cell Lines. The human breast cancer cell line, T-47D, obtained from EG and G Mason Research Institute, Rockville, MD, was derived from the pleural effusion of a patient with disseminated carcinoma of the breast (12). The cells were routinely maintained in DMEM supplemented with L-glutamine (4 mM), glucose (4.5 g/liter), penicillin (100 IU/ml), streptomycin (100 µg/ml), bovine insulin (10 µg/ml), and 10% (v/v) fetal bovine serum. This medium is also used for cell plating and will be referred to as PM. Trypsin-EDTA in HBSS was used for cell passages. Cells were maintained in a humid atmosphere of 95% air and 5% carbon dioxide at 37°C.

Hormones and Growth Factors. Purified human BSM was used for iodination. However, the BSM used for binding displacement and for growth studies was approximately 40 to 50% pure (1). Rat MSA II, a mixture of 4 polypeptides, and MSA III-2, a homogeneous preparation (19), were generous gifts of Dr. S. P. Nissley, NIH, CR-MSA was obtained from Collaborative Research, and is approximately the same as the preparation of Dulak and Temin (5). Purified bovine insulin (25.7 I.U./mg) was purchased from Grand Island Biological Co.

Iodination of Hormones. Basic SM was iodinated with Na125I using the chloramine-T method (11) as described previously (1). The specific radioactivities of 125I-BSM were 1900 cpm/fmol. Insulin and MSA III-2 were iodinated using lactoperoxidase (28). The specific radioactivity of 125I-insulin was 1250 cpm/fmol, and that of 125I-MSA III-2 was 1640 cpm/fmol.

Hormone Binding. To prepare cultures for binding studies, cells were seeded (4 x 10^6 cells/dish) in 35-mm plastic culture dishes in 2 ml of insulin-free PM and grown until monolayers were almost confluent (approximately 5 days after seeding). Growth medium was aspirated and cells were washed twice with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered HBSS containing 0.1% (w/v) bovine serum albumin (pH 7.4). All studies of hormone binding were performed with...
this buffer, and the incubation volume was 1 ml. The labeled ligand was either 125I-insulin, 125I-BSM, or 125I-MSA III-2. Incubation time and temperature were 60 min and 18°, respectively. The incubation was terminated by aspirating the radioactive medium and washing the monolayer 3 times with ice-cold phosphate-buffered saline. The cells were then dissolved with 1 ml of 3% sodium dodecyl sulfate, the solution transferred to disposable glass tubes, and the radioactivity determined in an LKB Wallac γ-counter with a counting efficiency of 70%. Specific binding was taken as the difference between the total radioactivity bound (mean of duplicates) in the absence of unlabeled hormone and that in the presence of 1 to 2 μg of unlabeled hormone. The latter represents nonspecific binding to cells and culture dishes. In Charts 1 to 4, the specific binding was expressed as a percentage of the total labeled hormone used.

Cell Growth on Plastic Substratum. T-47D breast cancer cells were plated in 35-mm dishes at a cell density 3 × 10^5 cell/dish in PM and incubated for 48 hr to allow for cell attachment. The medium was then discarded, and cells were washed twice with fresh serum-free insulin-free DMEM and replaced with the same medium. Test substances (BSM and CR-MSA) were added to dishes at different concentrations. Medium and hormones were changed on Days 3 and 6, and cell numbers were determined on Day 7. Cells were detached with trypsin-EDTA, dispersed in Isoton (Fisher), and counted in a Coulter Counter. For each determination, triplicate dishes were used.

Cell Growth on Collagen Gel. Collagen solution was prepared by mixing 9 parts of Vitrogen 100 (2.4 mg/ml) (Collagen Corp.), with 1 part of 10-fold-concentration DMEM as described by Leung and Shiu (15). One ml of this collagen solution was pipetted onto the bottom of each 35-mm culture dish. Gelation was completed after incubating the dish at 37° for 1 hr. T-47D cells, 3 × 10^5 cells/dish, were seeded in PM on top of the collagen gel and incubated for 2 days. The medium was aspirated, and the cells washed twice with serum-free DMEM and incubated for another 24 hr. This medium was again discarded and replaced with serum-free medium. The test substances were added at this time. Cell number was determined 6 days after the addition of hormones. In order to determine cell numbers, the collagen gel was digested with 0.1% collagenase (type III; Worthington) in HBSS for 1 hr at 37°. After centrifugation, the cells were further incubated for 5 min with 1 ml of trypsin/EDTA. Cells were dispersed in Isoton by pipeting and counted in a Coulter Counter. For each determination, triplicate dishes were used.

Statistics. Differences between 2 groups were analyzed using Student's t-test for 2 independent samples. Analysis of variance and Dunnett's test were followed when comparing multiple groups.

RESULTS

The optimal conditions for studying the binding of IGFs to the human breast cancer cells T-47D were first examined. Table 1 shows the results of temperature on specific binding of 125I-BSM to T-47D cells. The highest specific binding was observed between 15°-18°. Chart 1 shows the binding of 125I-BSM to T-47D cells at 18° were maximal at 1 hr. This was followed by a gradual decline upon further incubation.

Competitive Binding Studies of BSM, MSA, and Insulin. The binding of 125I-BSM and 125I-MSA III-2 to T-47D cells was determined. Chart 2 shows that when 125I-BSM was used, unlabeled BSM was most effective in displacing the ligand; 100 ng/ml of a partially purified preparation of unlabeled BSM produced 50% inhibition of 125I-BSM binding. Unlabeled insulin was weakly competitive, whereas CR-MSA did not displace the ligand.

Chart 3 shows the results of competitive binding between unlabeled insulin and the IGFs for 125I-MSA III-2 binding sites. MSA III-2 was most effective in displacing the labeled hormone; 40 ng/ml of unlabeled hormone produced 50% inhibition of 125I-MSA III-2 binding. Unlabeled BSM, MSA II, and insulin were weakly competitive, and concentrations as high as 1 µg/ml of these peptides did not produce 50% displacement.

The binding of 125I-insulin to T-47D was also studied. Chart 4 shows that MSA III-2 was more potent in displacing than unlabeled insulin. Half-maximum inhibition of 125I-insulin binding was produced by 10 ng/ml of unlabeled MSA III-2, whereas the same degree of inhibition was observed by 1 µg of insulin.

The effect of BSM and CR-MSA on the growth rate of T-47D cells on plastic is shown in Chart 5. When grown in serum-free medium BSM and CR-MSA were only weakly mitogenic to the cells; at 1 µg/ml, they produced less than a 2-fold increase in cell number. However, when T-47D were grown on collagen substratum (Chart 6), the mitogenic effect of BSM and MSA was readily apparent; cell proliferation was stimulated 3- to 4-fold when compared with control. When 3 different MSAs were compared (Chart 6), the purified MSA III-2 was the most potent peptide; it produced approximately 3.5-fold increase in cell number at a concentration of only 100 ng/ml. All the insulin-like growth peptides were more potent than insulin in stimulating T-47D cell growth.

DISCUSSION

In this study, the human breast cancer cell line T-47D has been shown to possess binding sites for BSM (IGF-I) and MSA III-2 (IGF-II). The results, however, suggest that the binding sites for BSM is distinct from the binding sites for MSA, since CR-MSA failed to displace 125I-BSM and since BSM only weakly displaced 125I-MSA III-2. Other studies show that, in chick embryo fibroblasts, MSA, IGF-I, and II appear to bind to the same receptor, whereas in human fibroblast and BRL-3A cells, there
is evidence for more than one receptor type (26). It appears therefore, that there are 2 types of binding sites for the IGFs present on T-47D cells; one specific for BSM (IGF-I) and the other for MSA (IGF-II). Thus, T-47D human breast cancer cells, similar to other cell types such as human placenta, BRL-3A cells, human fibroblasts (17, 26), possess both the type I (IGF-I) and type II (IGF-II) receptors.

125I-Insulin demonstrated low specific binding to T-47D cells confirming the recent results of Mountjoy et al. (21). The observation that both unlabeled MSA III-2 and MSA II inhibit the binding of 125I-insulin more than unlabeled insulin itself suggest that the mitogenic effect of insulin on the cells may be due, at least in part, to its binding to IGF-II receptors, rather than to insulin receptors. In addition, the finding that insulin at 1000 ng/ml displaces 125I-BSM and 125I-MSA III-2 to roughly the same extent (20%; Charts 2 and 3) suggests that insulin cross-reacts with both IGF-I and IGF-II receptors. Earlier studies using rat adipocytes have suggested that the common metabolic effects of insulin and IGFs are mediated through the insulin receptor, whereas the common growth-promoting effect is mediated by receptors for IGF (18, 25, 30). King et al. (13) demonstrated that the insulin receptor of rat adipocytes mediates the metabolic effects of insulin and MSA (IGF-II), whereas the growth-promoting actions of both peptides in human fibroblasts are mediated by either the IGF-I or the IGF-II receptor. Additional experiments involving cross-linking BSM, MSA, and insulin to T-47D cell membrane receptors are in progress and should help to clarify the nature of the receptors involved.

That the IGF binding sites in T-47D are functional receptors was illustrated by our finding that MSA and BSM are potent growth factors for the cells. It is of interest to note that BSM and MSA were only weakly mitogenic to T-47D cultured on a plastic substratum. Their mitogenic effects were more apparent when they were added to T-47D cells grown on collagen gel. This result was not surprising, since it has been shown previously that the physical substrate upon which epithelial cells rest in vitro and in vivo can modulate their response to growth factors. Gospodarowicz and Tauber (8) demonstrated that corneal epithelial cells maintained on plastic dishes failed to respond to epidermal growth factor, but those cells grown on collagen-coated dishes were stimulated to proliferate by epidermal growth factor. In view of the different responses to T-47D cells grown on collagen and ion plastic, it would be of interest to compare the IGF binding of these cells grown under the 2 conditions. In fact, we have attempted to measure the binding of 125I-IGF to T-47D cells grown on collagen. However, very high nonspecific binding to the collagen was encountered, and the specific binding had not been convincingly demonstrated.

Insulin was mitogenic to T-47D cells only when it was used at supraphysiological concentrations, probably due to its limited binding to the IGF receptors. The fact that epithelial cells respond to more than one of the IGFs is undoubtedly due in part to the structural similarities of the peptides. The IGFs are ancestrally related to proinsulin (5). Since MSA has been implicated as an important fetal growth factor (20) in the rat, the observation that human breast cancer cells responded to MSA may reflect the fetal characteristics of these cells acquired as a result of dedifferentiation associated with malignant transformation. The concentration of IGF-I and IGF-II in normal human serum were 150 to 250 and 800 to 1000 ng/ml, respectively (9). Our observation that 100 to 1000 ng/ml BSM (50% pure) and 10 to 1000 ng/ml MSA III-2 were active in stimulating T-47D cell growth indicate that these 2 peptides could be physiologically important growth factors for some breast cancer cells. Finally, the production of MSA/IGF-II is not known to be dependent on pituitary growth hormone but that of BSM/IGF-I is (31). This suggests that both pituitary-dependent and pituitary-independent pathways may be involved in influencing the growth of at least some forms of human breast cancer.

After the submission of this manuscript, Furlanetto and DiCarlo (7) reported that T-47D cells possess type I receptors, although type II receptors had not been studied. In addition, these investigators also found that insulin was weakly mitogenic to T-47D cells, and suggested that the mitogenic effect of insulin on these cells was mediated through a type I somatomedin receptor. Our results not only confirm these observations but also provide evidence to indicate that IGF-II is important in the growth regulation of human breast cancer cells.

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IGF AND HUMAN BREAST CANCER CELLS

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