Report

Mitogenic properties of insulin-like growth factors I and II, insulin-like growth factor binding protein-3 and epidermal growth factor on human breast epithelial cells in primary culture

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Summary

Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are growth factors implicated in mammary gland development and are believed to be involved in breast cancer. However, the interactions between components of the IGF system and breast epithelial cells, which give rise to breast cancer, are not well understood. We have investigated the mitogenic properties of IGF-I, IGF-II, IGF binding protein-3 (IGFBP-3) and epidermal growth factor (EGF) on human breast epithelial cells (HBEC) in primary culture. We show that, under serum-free conditions, HBEC are stimulated to grow in response to IGF-I and IGF-II in a dose-dependent manner. IGF-I and EGF, a potent stimulator of HBEC growth in primary culture and also associated with breast cancer, appear to stimulate HBEC in a synergistic manner. IGFBP-3 inhibits the stimulation by IGF-I, IGF-II and IGF-I plus EGF. In addition, it appears that IGFBP-3 has an inhibitory effect on HBEC growth that is IGF-independent. This study is the first to address the effects of IGF-I, IGF-II and IGFBP-3 alone and in combination with EGF on HBEC growth in primary culture. Characterizing the role of the IGF system in normal breast biology is significant because the system has been implicated in breast cancer and a number of the anti-estrogens used in treatment are believed to function through the IGF system.

Introduction

Current statistics indicate that one in nine women will be diagnosed with breast cancer during her lifetime and that ~50% of these women will die within 10 years of diagnosis. Although some progress has been made in the detection of early stage breast cancer and in the identification of important risk factors, as yet very little is understood about the cellular pathogenesis of this disease. We have developed a cell culture system in which we can routinely isolate and propagate human breast epithelial cells (HBEC) and stromal cells [1]. Using this system we have studied the effects of insulin-like growth factors (IGF) such as IGF-I, IGF-II and IGF binding proteins (IGFBP) on HBEC.

IGFs, classified as type I and type II, are polypeptides with structural homology to insulin. IGFs can elicit two types of biological responses: (1) rapid metabolic effects (insulin-like); and/or (2) slower growth promoting effects (mitogenic) [2]. Both IGF-I and IGF-II have been identified as potential risk factors for breast cancer. Recent population studies suggest an association between circulating IGF-I and breast cancer risk [3–5]. In a nested case-control study within the prospective Nurses' Health Study cohort, a positive correlation was observed between circulating IGF-I levels and breast cancer risk in premenopausal women [3]. A similar relationship was observed in two case-control studies [4, 5]. A number of studies have shown that breast cancer cell lines are responsive to both IGF-I and IGF-II [6–10]. Given that breast

epithelial cells give rise to breast cancer, we undertook this study to investigate the interaction between components of the IGF system and normal epithelial cells. The information gained will serve as a necessary baseline with which to compare data from experiments using cells from breast malignancies.

The bioactivity of IGFs is modulated by a number of components including: receptor activity, binding proteins, binding protein proteases, circulating levels and local production. IGFBP-3 is the major circulating IGFBP produced mainly in the liver; however, it is also produced locally by a number of tissues [11]. IGFBP-3 has been shown to inhibit IGF stimulation of growth and also to have IGF-independent growth inhibitory effects and induce apoptosis in breast cancer cell lines [12]. It has been demonstrated that the anti-proliferative effects of anti-estrogens in the MCF-7 cell line is associated with increased transcription of IGFBP-3 [13]. We have also investigated the IGF-dependent and IGF-independent effects of IGFBP-3 in normal HBEC.

Methods and materials

Dissociation procedure: Breast tissue was obtained from reduction mammoplasties. The tissue was dissociated into single cells and epithelial organoids and frozen for future use. Breast tissue dissociation protocols and cell culture were as previously described [14]. Briefly, tissue samples were trimmed of fat, minced, and dissociated by shaking at 37°C for ~18 h in a 1:1 mixture of Ham's F12:Dulbecco's modified Eagle's medium (F12:DME) supplemented with 10 mM Hepes buffer (H), 2% bovine serum albumin (BSA), 300 U/ml collagenase and 100 U/ml hyaluronidase. Following dissociation, the epithelial cell rich pellet was collected by centrifuging the cell suspension at $80 \times g$ for 4 min. Following resuspension of the epithelial pellet in a DMSO supplemented freezing medium, the cells were stored in liquid nitrogen until required.

Cell culture: Organoids were thawed and placed in non-tissue culture dishes overnight at 37°C in phenol-red free DME/F12/H with 5% fetal calf serum (FCS). Organoids were harvested and treated with saline-trypsin-EDTA to obtain a single cell suspension. Epithelial cells were resuspended in phenol-red free DME/F12/H with 5% FCS and seeded at 8000 cells/well in 96-well collagen-coated plates. On day 1, the medium was changed to serum-free (SF) medium

consisting of phenol-red free DME/F12/H, 1 mg/ml BSA, 0.5 µg/ml hydrocortisone (HC) and 10 ng/ml cholera toxin (CT), with or without the growth factors of interest (IGF-I, IGF-II and IGFBP-3, epidermal growth factor (EGF)). Thereafter, the medium was changed on alternate days. When cultures were approximately 80% confluent, they were terminated and optical densities were determined using the tetrazolium dye (MTT) reduction assay [15].

Immunohistochemistry: The alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure (DAKO EnVision system K4017) was used to stain cytospun cells or cultured cells in situ. Primary antibodies used were to epithelial specific antigen (ESA) (Vector), cytokeratin 19 (CK19) (Dako) and common acute lymphoblastic leukemia antigen (CALLA) (DAKO).

MTT assay: Culture medium was removed and medium (phenol-red-free DME/F12/H) containing 1 mg/ml MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) was added to each well in a volume of 100 µl and incubated in 95% air and 5% CO₂ at 37°C for 5 h. Afterwards, 100 µl of 20% formol in saline was added to each well for 30 min at room temperature. The medium was removed and 100 µl of anhydrous isopropanol was added to each well for 1 h at room temperature. A 96-well microtiter plate reader (model EL 311, Biotek Instruments) was used to determine absorbance values at 540 nm. Optical densities were converted to a percentage of control (cultures incubated in medium without any growth factors).

Results

Isolation and propagation of HBEC: We collected tissue from reduction mammoplasties and performed enzymatic and mechanical dissociation which resulted in a mixed cell population of epithelial and stromal cells (see Methods and materials). This mixed cell population was subjected to differential centrifugation in order to separate the epithelial and stromal cell components. The epithelial cell component was then used for the experiments outlined in this report. In order to ensure that we were culturing epithelial cells, cytospun preparations of cell suspensions used in growth experiments, as well as the cultured cells were stained using antibodies to two epithelial cell markers: ESA, which identifies epithelial cells, and CK19, which identifies luminal epithelial cells (Figure 1). The cultured cells were also stained with CALLA which recognizes

Figure 1. IHC staining of HBEC used in growth experiments. (A) Expression of CK19 in cytospun cells from epithelial cell-enriched suspension of freshly dissociated human mammary organoids. (B) Expression of CK19 in HBEC cultured in SF medium for 7 days.

fibroblasts and myoepithelial cells. A minority of the cells stained with CALLA (data not shown).

Stimulation of HBEC growth in primary culture by IGF-I and IGF-II: HBEC were cultured in serum-free conditions to examine the effects of IGF- I and IGF-II at three concentrations: 1, 10 and 100 ng/ml. Cultures were maintained for approximately 6 days when they were 80% confluent. At the end of the culture period

an MTT assay was performed. Figures 2(A) and (B) are representative graphs demonstrating that both IGF-I and IGF-II stimulate growth in a dose-dependent manner ($p < 0.05$; $n = 5$). Figure 3 demonstrates that physiological doses of IGF-I (10 and 100 ng/ml) stimulate primary HBEC to a similar or greater extent than a pharmacological dose of insulin (1.0 ug/ml) ($p < 0.05$; $n = 3$). When IGF-I (10 ng/ml) and insulin

Figure 2. (A) IGF-I stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence or absence of IGF-I (1, 10 or 100 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGF-I (10 or 100 ng/ml) significantly stimulated cell growth above control levels ($p < 0.05$; $n = 5$). (B) IGF-II stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence or absence of IGF-II (1, 10 or 100 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGF-II (10 or 100 ng/ml) significantly stimulated cell growth above control levels ($p < 0.05$; $n = 5$).

Figure 3. IGF-I and insulin stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence of a pharmacological concentration of insulin (1 ug/ml) or physiological concentrations of IGF-I (10 or 100 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). Physiological levels of IGF-I stimulated HBEC cell growth to similar or greater levels than pharmacological levels of insulin ($p < 0.05$; $n = 3$).

(1.0 ug/ml) are added to the medium together, IGF-I has no effect (data not shown).

IGFBP-3 inhibition of growth stimulation of HBEC by IGF-I and IGF-II: IGFBP-3 is the major circulating IGFBP, which is also produced locally in tissues. We examined the effects of recombinant IGFBP-3 (5–500 ng/ml) on the growth of HBEC in primary culture in the presence of IGF-I (10 ng/ml) or IGF-II (10 ng/ml). Both IGFBP-3 and the growth factor of interest were added simultaneously to the culture medium. Cultures were maintained for approximately 6 days when they were 80% confluent. At the end of the culture period an MTT assay was performed. IGFBP-3 showed a dose-dependent inhibition of growth stimulation by IGF-I ($p < 0.05$; $n = 4$; Figure 4). Similarly, IGFBP-3 inhibited IGF-II stimulation in a dose-dependent manner ($p < 0.05$; $n = 4$) (data not shown).

IGF-independent effects on growth of HBEC by IGFBP-3: Current data from laboratories utilizing human breast cancer cell lines, including MCF-7, suggest that IGFBP-3 has effects on cell growth independent of IGF-I. We examined these effects on HBEC in primary culture and on the established MCF-7 breast cancer cell line. We grew HBEC in primary culture and MCF-7 cells in the presence and absence

of IGFBP-3. Cultures were maintained for approximately 6 days when they were 80% confluent. At the end of the culture period an MTT assay was performed. Figure 5(A) demonstrates that IGFBP-3 decreased cell growth in MCF-7 cells in the presence of 0.5% FCS ($p < 0.05$; $n = 3$), while Figure 5(B) demonstrates that similar IGF-independent inhibition of growth by IGFBP-3 was observed in HBEC in SF conditions ($p < 0.05$; $n = 3$).

Stimulation of HBEC growth by IGF-I and EGF: EGF is a potent stimulator of breast epithelial cell growth. We examined the growth of HBEC in primary culture in the presence of EGF and IGF-I, alone and in combination. We grew HBEC under SF conditions in the presence of either (a) EGF alone (1 or 10 ng/ml); (b) IGF-I alone (10 ng/ml) or (c) EGF (1 or 10 ng/ml) plus IGF-I (10 ng/ml). Cultures were maintained for approximately 6 days when they were 80% confluent. At the end of the culture period an MTT assay was performed. Figure 6(A) demonstrates that EGF (1 or 10 ng/ml) plus IGF-I (10 ng/ml) stimulates HBEC growth greater than either growth factor alone ($p < 0.05$; $n = 3$).

Inhibitory effects of IGFBP-3 on HBEC growth in the presence of IGF-I and EGF: We examined the effects of recombinant IGFBP-3 (50, 150, 400,

Figure 4. Effects of IGFBP-3 on IGF-I stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence of IGF-I (10 ng/ml) with or without IGFBP-3 (5–500 ng/ml) for approximately 6 days until they were 80% confluent. IGF-I and IGFBP-3 were added to the culture media at the same time. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGFBP-3 inhibited cell growth in a dose-dependent manner ($p < 0.05$; $n = 3$).

500 ng/ml) on the growth of HBEC in primary culture in the presence of IGF-I (10 ng/ml) and EGF (10 ng/ml). Both IGFBP-3 and the growth factors were added simultaneously to the culture medium. Cultures were maintained for approximately 6 days when they were 80% confluent. At the end of the culture period an MTT assay was performed. IGFBP-3 showed a dose-dependent inhibition of stimulation by IGF-I ($p < 0.05$; $n = 3$; Figure 4). Figure 6(B) is a representative graph indicating that IGFBP-3 also inhibits HBEC growth stimulation by EGF and IGF-I in a dose-dependent manner ($p < 0.05$; $n = 3$).

IGF-independent effects of IGFBP-3 on HBEC in the presence of EGF: We examined the IGF-independent effects of IGFBP-3 on HBEC in primary culture in the presence of EGF. We grew HBEC in SF conditions in the presence of IGFBP-3 (50, 150, 400, 500 ng/ml) and EGF (10 ng/ml). Both IGFBP-3 and EGF were added simultaneously to the culture medium. Cultures were maintained for approximately 6 days when they were 80% confluent. At the end

of the culture period an MTT assay was performed. Figure 6(C) demonstrates that IGFBP-3 decreased cell growth in HBEC in a non-dose-dependent manner when EGF is present in the culture media ($p < 0.05$; $n = 3$).

Discussion

In this study, we examined the effects of IGF-I, IGF-II, IGFBP-3 and EGF on HBEC in primary culture. We demonstrate that (a) IGF-I and IGF-II stimulate primary HBEC in a dose-dependent manner; (b) physiological concentrations of IGF-I stimulate primary HBEC to a similar or greater extent than pharmacological concentrations of insulin; (c) EGF and IGF-I added to the culture medium together stimulate HBEC growth in a synergistic manner; (d) the stimulatory effects observed in (a) and (c) above can be blocked by the addition of IGFBP-3 to the culture

Figure 5. (A) IGF-independent effects of IGFBP-3 on MCF-7 cell growth. MCF-7 were cultured in 0.5% FCS in the presence or absence of IGFBP-3 (50, 150 or 300 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (0.5% FCS condition). IGFBP-3 significantly inhibited cell growth ($p < 0.05$; $n = 3$). (B) IGF-independent effects of IGFBP-3 on HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence or absence of IGFBP-3 (5–500 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGFBP-3 significantly inhibited cell growth in a dose-dependent manner ($p < 0.05$; $n = 3$).

medium; (e) IGFBP-3 can inhibit the growth of HBEC in an IGF-independent manner.

Using our culture system, we routinely propagate HBEC in primary culture. We have confirmed the epithelial nature of the cultured cells by staining with ESA and CK 19 (see Results). To our knowledge, this is the first report examining the mitogenic effects of IGF-I, IGF-II, IGFBP-3 alone and in combination with EGF on HBEC in primary culture.

Our demonstration that IGF-I and IGF-II have direct effects on HBEC in primary culture, stimulating growth in a dose-dependent manner, serves as a baseline for future examination of breast neoplasms. In population studies, circulating IGF-I levels have been identified as potential breast cancer risk factors [3–5]. In addition, Ng et al. [16] demonstrated that circulating IGF-I levels are correlated with mammary gland proliferation. Our results show a direct

Figure 6. (A) IGF-I and EGF stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence of IGF-I (10 ng/ml) and/or EGF (1 or 10 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGF-I (10 ng/ml) alone and EGF (1 or 10 ng/ml) alone significantly stimulated cell growth above control levels ($p < 0.05$; $n = 3$). IGF-I (10 ng/ml) plus EGF (1 or 10 ng/ml) significantly stimulated cell growth above control levels and above either growth factor alone ($p < 0.05$; $n = 3$). (B) Effects of IGFBP-3 on EGF and IGF-I stimulation of HBEC growth in primary culture. HBEC were cultured in SF conditions in the presence of IGF-I (10 ng/ml) plus EGF (10 ng/ml) with or without IGFBP-3 (50–500 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGF-I (10 ng/ml) plus EGF (10 ng/ml) significantly stimulated cell growth above control levels ($p < 0.05$; $n = 3$), while the addition of IGFBP-3 inhibited this stimulation in a dose-dependent manner ($p < 0.05$; $n = 3$). (C) IGF-independent effects of IGFBP-3 on HBEC growth in the presence of EGF. HBEC were cultured in SF conditions in the presence of EGF (10 ng/ml) with or without IGFBP-3 (50–500 ng/ml) for approximately 6 days when they were 80% confluent. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). EGF (10 ng/ml) alone significantly stimulated cell growth ($p < 0.05$; $n = 3$). The addition of IGFBP-3 significantly inhibited cell growth in a non-dose-dependent manner ($p < 0.05$; $n = 3$).

interaction between IGF-I or IGF-II and normal HBEC. We also demonstrate that physiological concentrations of IGF-I are as potent or more potent than pharmacological levels of insulin in stimulating the growth of HBEC. Conversely we demonstrate that if

pharmacological levels of insulin are present in culture media, IGF-I does not have any effect (data not shown). This may be significant not only for cancer therapies but also for treatment protocols using growth hormone and/or IGF-I for other growth disorders.

Figure 6. (continued)

Previous reports have demonstrated that IGFBP-3 has IGF-independent effects in breast cancer cell lines including MCF-7 and Hs578T [12, 17]. In this study, we examined the effects of IGFBP-3 on HBEC growth in primary culture and MCF-7 cells in the absence of IGF-I and IGF-II. IGF-independent IGFBP-3 inhibition of cell growth was observed in both. This finding suggests that IGF-independent IGFBP-3 growth inhibitory effects are part of normal breast biology and not necessarily a result of cell transformation and/or immortalization. In addition, we demonstrated that, when EGF and IGFBP-3 were simultaneously added to the culture medium, IGFBP-3 inhibited cell growth. This may be of great interest clinically because a number of breast cancer treatment strategies attempt to block the EGF signal transduction pathway, such as Herceptin [18].

This study has been the first to address the effects of IGF-I, IGF-II and IGFBP-3 alone and in combination with EGF on HBEC growth in primary culture. Given the fact that a number of the anti-estrogens used in the treatment of breast cancer are believed to function through the IGF system [19], it is important to characterize the role of the IGF system, both growth factors and binding proteins in normal breast biology. The antiestrogen Tamoxifen, initially used in the palliative treatment of advanced breast cancer, is now commonly used in adjuvant therapy. Furthermore, current clinical trials in North America and Europe are investigating the chemopreventive effects of Tamoxifen in women at high risk for breast cancer [20–22]. Tamoxifen has been shown to reduce cir-

culating levels of IGF-I while increasing circulating levels of IGFBP-1 and IGFBP-3 [23]. It also affects local IGF production in several tissues including the endometrium [24]. There is evidence that Tamoxifen can modulate IGF signal transduction [25]. Characterizing the role of the IGF system, both growth factors and binding proteins, in normal breast biology should help us understand the therapeutic value of Tamoxifen.

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