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 stromal cells in primary culture

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Summary

Insulin-like growth factors I and II (IGF-I and IGF-II) are growth factors implicated in both normal mammary gland development and breast cancer. We have previously reported on the effects of components of the IGF system on breast epithelial cells. Since data suggests that stromal–epithelial interactions play a crucial role in breast cancer, we have now investigated the mitogenic properties of IGF-I, IGF-II, insulin-like growth factor binding protein-3 (IGFBP-3) and epidermal growth factor (EGF) on human breast stromal cells in primary culture. We show that, under serum-free conditions, stromal cells 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 human breast epithelial cell growth in primary culture and also associated with breast cancer, appear to stimulate stromal cell growth in a synergistic manner. IGFBP-3 does not inhibit the stimulation of growth by IGF-I, or IGF-I plus EGF. However, IGFBP-3 does inhibit the stimulation of growth by IGF-II. In contrast to our previous results with human breast epithelial cells, IGFBP-3 does not have an IGF-independent inhibitory effect on stromal cell growth. This study is the first to address the effects of IGF-I, IGF-II and IGFBP-3 alone and in combination with EGF on human breast stromal cell growth in primary culture. Characterizing the role of the IGF system in both normal breast epithelial cells and stromal cells will aid in our understanding of the mechanisms behind the role of the IGF system in breast cancer.

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 in primary culture [1]. Utilizing this culture system, we have previously reported the effects of insulin-like growth factors I and II (IGF-I, IGF-II), insulin-like growth factor binding protein-3

(IGFBP-3) and epidermal growth factor (EGF) on the epithelial cells. Since stromal–epithelial interactions are important in the normal breast development, we now report on the effects of components of the IGF system on the stromal cells.

Insulin-like growth factors (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]. In addition, there appears to be a relationship between (1) IGF-I gene expression and mammographic densities and (2) IGF-I serum levels and mammographic densities [6]. Mammographic density has been identified as a strong risk factor for breast cancer [7]. Stromal tissue, comprised of fibroblasts, adipocytes, extracellular matrix and vascular components, surrounds the epithelium. Both tissues are reflected on a mammogram as radiologic densities.

Messenger RNA analysis of breast cancer cell lines and *in situ*/immunohistochemical analysis of primary breast tumors suggest that IGF-I is produced by stromal cells and IGF-II is produced by both stromal and epithelial cells [8–14]. 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 IGF binding protein produced mainly in the liver; however, it is also produced locally by a number of tissues [15]. In a previous report [16], we observed that IGFBP-3 inhibits IGF-I and IGF-II stimulation of HBEC growth and also has IGF-independent growth inhibitory effects. This study was designed to examine the effects of IGF-I and IGF-II on breast stromal cell proliferation and the potential inhibitory effects of IGFB-3, both IGF-dependent and independent.

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 and cell culture protocols were as previously described [17]. 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 resulting cell suspension was centrifuged several times, with washes, at $80 \times g$ for 4 min, to remove the majority of epithelial cells and organoids. The remaining cells were pelleted by centrifugation at $100 \times g$ for 5 min.

This stromal-cell enriched pellet was resuspended in DMSO-supplemented freezing medium and stored in liquid nitrogen until required.

Cell culture. Stromal cell pellets were thawed, washed and viable cells counted. The cells were resuspended in phenol red free DME/F12/H with 5% fetal calf serum and seeded at 16,000 cells per well in 96-well tissue culture plates; 10^6 cells were also placed in a 25 cm² tissue culture flask. After 2 h incubation at 37°C in 5% CO₂ to allow attachment of fibroblasts, the growth medium was removed, the attached cells gently rinsed, and fresh growth medium added to the wells and flask. After 24 h incubation, the medium was replaced with a serum-free medium consisting of phenol red free DME/F12/H, 1 mg/ml BSA, 1 ng/ml fibroblast growth factor, with or without the growth factors of interest (IGF-I, IGF-II, EGF, IGFBP-3). Thereafter, the medium was changed on alternate days. After 6 days, the cultures were terminated and growth determined by measuring optical densities using the tetrazolium dye (MTT) reduction assay [18].

Immunohistochemistry. The alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure (DAKO EnVision system K4017) was used to stain cytospun cells harvested from the 25 cm² tissue culture flasks at the time of the MTT assay. Primary antibodies used to identify these cells included those against vimentin (V9; Dako), cytokeratins (AE1/AE3, Dako), epithelial specific antigen (ESA; Vector) and Von Willibrand Factor 8 (Dako). Positive controls included sections of human breast tissue stained with these three antibodies; as a negative control, an anti-dextran monoclonal antibody was used with both tissue sections and cytospun cultured cell preparations.

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 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.

Figure 1. IHC staining of human breast stromal cells used in growth experiments. Cells cultured for 7 days in serum-free conditions (SF), cytopun and stained with antibodies to (A) vimentin, (B) cytokeratins (AE1/AE3) or (C) Factor 8. Very few keratin-positive (epithelial) or Factor 8-positive (endothelial) cells were detected. Positive staining of appropriate cells in human breast tissue with these three antibodies (vimentin, cytokeratins and factor 8) is shown (D), (E) and (F)). No staining was seen using an anti-dextran monoclonal antibody (negative control; (G)).

Figure 2. Effects of IGF-I and IGF-II on human breast stromal cell growth in primary culture. Human breast stromal cells were cultured in SF in the presence or absence of IGF-I (10 or 100 ng/ml) or IGF-II (1, 10 or 100 ng/ml) for approximately 6 days. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). (a) IGF-I (10 or 100 ng/ml) significantly stimulated cell growth above control levels ($p < 0.05$; $n = 5$). Similarly, (b) shows that IGF-II (10 or 100 ng/ml) significantly stimulated cell growth above control levels ($p < 0.05$; $n = 3$).

Optical densities were converted to a percentage of control (cultures incubated in medium without any growth factors).

Statistical analysis. An ANOVA test was performed on all growth experiments. For post-hoc analysis a Tukey's test was utilized.

Results

Isolation and propagation of human breast stromal cells

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 and differential attachment to tissue culture plastic in order to separate the epithelial and stromal cell components. The stromal cell component was then used for the experiments outlined in this report. In order to ensure that the majority of cells we were culturing were stromal cells (as opposed to epithelial cells), cytospun preparations of cultured cells were stained using antibodies against epithelial and stromal markers: cytokeratins (AE1/AE3), ESA, vimentin (V9), and Von Willibrand Factor 8 (present in endothelial cells). The vast majority of cells cultured were cytokeratin-, ESA- and Factor 8-negative and vimentin positive (Figure 1). As well, microscopic morphology of the cells in culture was fibroblastic.

Stimulation of breast stromal cell growth in primary culture by IGF-I and IGF-II

Human breast stromal cells were cultured in serum-free conditions to examine the effects of IGF-I and IGF-II at two concentrations: 10 and 100 ng/ml. Cultures were maintained for 6 days. At the end of the culture period an MTT assay was performed. Figure 2(a) and (b) are representative graphs demonstrating that both IGF-I and IGF-II stimulate growth in a dose-dependent manner ($p < 0.05$; $n = 5$).

IGFBP-3 inhibition of growth stimulation of HBEC by IGF-I or IGF-II

IGFBP-3 is the major circulating IGF binding protein, which is also produced locally in tissues. We examined the effects of recombinant IGFBP-3 (5–500 ng/ml) on the growth of human breast stromal cells in primary culture in the presence of IGF-I or IGF-II (10 and 100 ng/ml). Both IGFBP-3 and IGF-I or IGF-II were added simultaneously to the culture medium. Cultures were maintained for 6 days. At the end of the culture period an MTT assay was performed. IGFBP-3 did not inhibit growth stimulation by IGF-I ($p < 0.05$; $n = 6$; Figure 3(a)). However, it does inhibit growth stimulation by IGF-II ($p < 0.05$; $n = 3$; Figure 3(b)).

IGF-independent effects on growth of human breast stromal cells by IGFBP-3

In a previous report [16], we demonstrated that IGFBP-3 inhibited human breast epithelial cell growth independent of IGF-I. We examined these effects on breast stromal cells in primary culture. We grew human breast stromal cells in primary culture in the

Figure 3. Effects of IGFBP-3 on IGF-I and IGF-II stimulation of human breast stromal cell growth in primary culture. Human breast stromal cells were cultured in serum-free conditions (SF) in the presence of IGF-I (10 or 100 ng/ml) or IGF-II (100 ng/ml) with or without IGFBP-3 (50–500 ng/ml) for approximately 6 days. IGF-I or IGF-II and IGFBP-3 were added to the culture medium 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 did not inhibit IGF-I stimulated cell growth ($n = 6$; (a)); in contrast, IGFBP-3 did inhibit IGF-II stimulated cell growth ($p < 0.05$; $n = 3$; (b)).

Figure 4. IGF-independent effects of IGFBP-3 on human breast stromal cell growth in primary culture. Human breast stromal cells were cultured in serum-free conditions in the presence or absence of IGFBP-3 (50–500 ng/ml) for approximately 6 days. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGFBP-3 alone did not inhibit cell growth ($n = 6$).

presence and absence of IGFBP-3. Cultures were maintained for 6 days. At the end of the culture period an MTT assay was performed. Figure 4 demonstrates that IGFBP-3 does not demonstrate IGF-independent inhibition of growth in serum-free conditions ($p < 0.05$; $n = 6$).

Stimulation of human breast stromal cell growth by IGF-I and EGF

We examined the growth of human breast stromal cells in primary culture in the presence of IGF-I and EGF, alone and in combination. We grew human breast stromal cells under serum-free conditions in the presence of either (a) IGF-I alone (10 ng/ml), or (b) EGF alone (1 or 10 ng/ml) or (c) IGF-I (10 ng/ml) plus EGF (1 or 10 ng/ml). Cultures were maintained for 6 days. At the end of the culture period an MTT assay was performed. Figure 5 demonstrates that IGF-I (10 ng/ml)

plus EGF (1 or 10 ng/ml) stimulates human breast stromal cell growth greater than either growth factor alone ($p < 0.05$; $n = 6$).

plus EGF (1 or 10 ng/ml) stimulates human breast stromal cell growth greater than either growth factor alone ($p < 0.05$; $n = 6$).

Inhibitory effects of IGFBP-3 on human breast stromal cell growth in the presence of IGF-I and EGF

We examined the effects of recombinant IGFBP-3 (50, 150 ng/ml) on the growth of human breast stromal cells 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 6 days. At the end of the culture period an MTT

Figure 6. Effects of IGFBP-3 on IGF-I and EGF stimulation of human breast stromal cell growth in primary culture. Human breast stromal cells were cultured in serum-free conditions (SF) in the presence of IGF-I (10 ng/ml) and/or EGF (1 or 10 ng/ml) with or without IGFBP-3 (50 or 150 ng/ml) for approximately 6 days. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGFBP-3 (50 or 150 ng/ml) did not inhibit the stimulatory effects of IGF-I (10 ng/ml) plus EGF (1 or 10 ng/ml) ($n = 3$).

assay was performed. IGFBP-3 did not inhibit stimulation of stromal cell growth by IGF-I and EGF ($n = 3$; Figure 6).

IGF-independent effects of IGFBP-3 on human breast stromal cells in the presence of EGF

We examined the IGF-independent effects of IGFBP-3 on human breast stromal cells in primary culture in the presence of EGF. We grew human breast stromal cells in serum-free 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 6 days. At the end of the culture period an MTT assay was performed. Figure 7 demonstrates that IGFBP-3 had

Figure 7. Effects of IGFBP-3 on EGF stimulation of human breast stromal cell growth in primary culture. Human breast stromal cells were cultured in serum-free conditions (SF) in the presence of EGF (1 or 10 ng/ml) with or without IGFBP-3 (50, 150 ng/ml) for approximately 6 days. The MTT assay was used to assess cell growth. Results are expressed as a percentage of the control condition (SF condition). IGFBP-3 did not inhibit the stimulation of human breast stromal cell growth by EGF ($n = 4$).

no effect on cell growth in human breast stromal cells with EGF present in the culture media ($n = 4$).

Discussion

In this study, we examined the effects of IGF-I, IGF-II, IGFBP-3 and EGF on human breast stromal cells in primary culture. We demonstrate that (a) IGF-I and IGF-II stimulate human breast stromal cells in a dose-dependent manner; (b) IGF-I and EGF added to the culture medium together stimulate human breast stromal cell growth in an additive manner; (c) IGFBP-3 does not inhibit the stimulatory effects of IGF-I and IGF-I plus EGF in human breast stromal cells; however, IGFBP-3 does inhibit IGF-II stimulation of human breast stromal cells; and (d) unlike our observations in human breast epithelial cells, IGFBP-3 does not inhibit the growth of human breast stromal cells in an IGF-independent manner.

Using our culture system, we routinely propagate human breast stromal cells in primary culture. We have confirmed the stromal (non-epithelial) nature of the cultured cells by staining with antibodies to cytokeratins, vimentin and Von Willibrand Factor 8. To our knowledge, this is the first report examining the mitogenic effects of IGF-I, IGF-II, and IGFBP-3 alone and in combination with EGF on human breast stromal cells in primary culture.

In population studies, circulating IGF-I has been identified as a potential breast cancer risk factor [3–5]. In addition, there appears to be a correlation between IGF-I gene expression and IGF-I serum levels with mammographic densities [6]. There is now evidence of a relationship between mammographic density and breast cancer [7]. Our results show a direct interaction between IGF-I or IGF-II and normal human breast stromal cells. In a previous paper [16] we reported that IGF-I and IGF-II stimulated growth of epithelial cells. Therefore, IGFs can stimulate growth of both epithelial and stromal cells. Since stromal–epithelial interactions are implicated in tumor development as well as normal breast development, our demonstration that IGF-I and IGF-II have direct effects on the stromal cells as well as the epithelial cells, serves as a baseline for future examination of breast neoplasms.

Interestingly, we demonstrated that IGFBP-3, a protein known to inhibit IGF-I and IGF-II stimulation in epithelial cells, does not have any effect on IGF-I stimulation in stromal cells. It does however inhibit IGF-II stimulation. In addition, we have demonstrated

that IGFBP-3 does not inhibit stimulation of stromal cells by IGF-I + EGF. This is in contrast to the effect observed in epithelial cells [16]. This suggests that there are different mechanisms involved in (a) the interaction between IGFBP-3 and epithelial cells and that between IGFBP-3 and stromal cells, and (b) the interaction between IGFBP-3 and IGF-I and that between IGFBP-3 and IGF-II.

We have previously reported that IGFBP-3 has IGF-independent effects in human breast epithelial cells [16]. We and others have reported similar effects on breast cancer cell lines including MCF-7 and Hs578T [16, 19, 20]. In this study we examined the effects of IGFBP-3 on human breast stromal cell growth in primary culture in the absence of IGF-I and IGF-II. In contrast to data on epithelial cells, IGF-independent IGFBP-3 does not inhibit cell growth in the stromal cells. In addition, we demonstrated that, when EGF and IGFBP-3 is simultaneously added to the culture medium, IGFBP-3 does not inhibit cell growth. This is in contrast to effects observed in epithelial cells where IGFBP-3 inhibits EGF stimulation of epithelial cells [16]. Again, these results suggests that IGFBP-3 may exert its effects via different mechanisms in epithelial cells and stromal cells.

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 human breast stromal cell growth in primary culture. Given the fact that stromal-epithelial interactions are important in tumor development [21], it is important to characterize the role of the IGF system, both growth factors and binding proteins, in normal breast stromal cells as well as epithelial cells.

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