PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 70 (1999) 249–256

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Effects of matrix components on aromatase activity in breast stromal cells in culture

Anne L. Quinn[a], William E. Burak Jr.[b,c], Robert W. Brueggemeier[a,c,d,*]

[a]*Ohio State Biochemistry Program, The Ohio State University, Columbus, OH 43210, USA*
[b]*Department of Surgery, College of Medicine, The Ohio State University, Columbus, OH 43210, USA*
[c]*OSU Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA*
[d]*College of Pharmacy, Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, USA*

Received 16 September 1998; accepted 31 March 1999

Abstract

Local estradiol production within breast tissue is maintained by the aromatase cytochrome $P450_{arom}$ complex, which has been localized primarily to the stromal component of tumors but also has been detected in the breast epithelial cells. Paracrine interactions between stromal and epithelial components of the breast are critical to the sustained growth and progression of breast tumors. Maintenance of the differentiated state, including hormone and growth factor responsiveness, requires extracellular matrix proteins as substrata for cells. This research has focused on developing a cell culture system that more closely mimics in vivo interactions in order to dissect actual paracrine signaling between these two cell types. Human fibroblasts were isolated from breast tissue and were maintained in a cell culture system grown on plastic support or on a collagen I support matrix. The collagen I matrix model supports cell maintenance and subsequent differentiation on collagen rather than maximal proliferation, therefore allowing for a more accurate environment for the study of hormonal control and cellular communication. Initial experiments compared aromatase activity of patient fibroblasts grown on plastic versus collagen I using the tritiated water release method. Constitutive aromatase activity was found to be lower when cells were grown on a collagen gel for 4–7 days (7.7 fold lower) using DMEM/F12 containing 10% dextran coated charcoal stripped serum. However, fibroblasts grown on collagen I appeared to be significantly more responsive to stimulation by 100 nM dexamethasone (plastic: 6.0 fold induction, collagen: 33.2 fold induction) when pretreated for 12 h prior to measurement of aromatase activity. In an effort to examine paracrine interactions between the stromal and epithelial cells in breast tissue, experiments using conditioned media from fibroblast cultures were performed. Testosterone administration to fibroblasts results in the production of estradiol into the media in sufficient concentrations to elicit an increase in pS2 expression when the conditioned media is administered to MCF-7 cells. The addition of a potent aromatase inhibitor resulted in a complete suppression of fibroblast-derived estrogens and showed only a modest increase in pS2 expression. Culturing breast fibroblasts and epithelial cells on extracellular matrix allows for a more meaningful examination of the paracrine interactions between these cell types within the context of an appropriate extracellular environment. This study highlights the need for evaluation of gene expression in cell culture systems that accurately reflect the tissue microenvironment.

1. Introduction

Approximately two-thirds of newly diagnosed breast cancer in the U.S. is hormone-dependent breast cancer, requiring estrogen for tumor growth. Estrogen is considered important for the local stimulation of growing malignancies in the breast. Estradiol, the most potent endogenous estrogen, is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. The highest levels of aromatase enzymatic activity are present in the ovaries of premenopausal women, in the placenta of pregnant women and in the peripheral adipose tissues of postmenopausal women

* Corresponding author. Tel.: +1-614-292-5231; fax: +1-614-292-2435.
E-mail address: brueggemeier.1@osu.edu (R.W. Brueggemeier)

0960-0760/99/$ - see front matter
PII: S0960-0760(99)00113-2

and of men. Aromatase activity has also been demonstrated in breast tissue in vitro [1–4]. Furthermore, expression of aromatase is highest in or near breast tumor sites [2,5].

Estrogens exhibit many functions that include regulation of growth, proliferation and differentiation as well as metabolic processes. Many of these events are estrogen receptor mediated through the complex regulation of key genes that are expressed in a well controlled temporal and tissue specific pattern [6]. Paracrine interactions, including cell-cell mediated communication, are an important physiological process by which surrounding cells can modulate other cells' activity and maintain proper tissue integrity. Normal tissue communication can become dysregulated in the presence of malignancies. Tumor cells exhibit insensitivity to regular cellular cues and setup an environment that will maximize tumor growth. Tumor derived factors may stimulate expression of other factors which may normally not be expressed and also tumors cells fail to respond to contact inhibition, normally a signal to halt cellular growth [7]. An additional level of organization is provided by the surrounding extracellular matrix which provides the three dimensional framework upon which the tissue is structured. These extracellular matrix proteins interact via specific receptor mediated events and alter transcriptional activity of the cell [8].

The extracellular matrix (ECM) generally consists of at least 50 different proteins, which provide a framework of tissue throughout the body [9]. In the breast, the basement membrane, which is a specialized form of the extracellular matrix, surrounds epithelial cells and other cell types and is primarily composed of laminin, type IV collagen and proteoglycans. The interstitial tissue matrix is made up of structural ECM proteins, in addition to the stromal fibroblasts and the adipocytes. The interstitial matrix of breast tissue is composed primarily of collagen I and some fibronectin [10]. The role of the ECM appears to be more complex than simply providing physical structure to the tissue. Expression of specific genes is highly dependent on receiving coordinated extracellular signals. These signals represent a cooperation of extracellular matrix signals with other regulatory molecules such as hormones and growth factors.

The extracellular matrix proteins generally exert their effect through transmembrane receptors, usually from the integrin family [11]. The integrin receptors are made up of an α and β chain; a wide variety of specific receptors can be formed through variable association of these α and β subunits. These receptors generally have short cytoplasmic domains which interact directly with cytoskeletal α-actinin and talin. Generally, the cytoskeleton controls the shape of the cell, the motility and gene expression [9]. The major mechanism responsible for re-organizing the cytoskeleton is the polymerization and depolymerization of the microtubules. It has been hypothesized that the alterations in the cytoskeleton may be central to the acquisition or loss of a differentiated phenotype [13]. The exact link between ECM interaction with the integrin receptor and alterations in gene expression is tentative. In one model study, laminin provided an environment where cultured mammary epithelial cells produced β casein when stimulated with prolactin. The subsequent addition of colchicine was able to inhibit the production of β casein and also the transcription of the mRNA [14]. Jackson and Cook showed in the early 1980s that the nuclear matrix was commonly associated with DNA being replicated and transcribed. They suggested that the nuclear matrix was one part of the active site of the transcription complex [15,16].

The collagen family of proteins is composed of 14 different types representing homo- and heterotrimeric complexes showing a wide range of structures and functions [12]. Collagen I is a heterotrimeric helix of $\alpha_1(I),\alpha_2(I)_1$.These complexes are synthesized as propeptides and assembled extracellularly into crosslinked fibrils. The collagen I receptor is the integrin VLA-2 (α2,β1) transmembrane receptor. This receptor is commonly expressed on the surface of several cell types, such as fibroblasts, breast epithelial cells and endothelial cells. Collagen I has been shown to be a very potent modulator of cellular function [17]. In vitro studies have shown that growing cells on a collagen I substratum can lead to alterations in rates of proliferation, adhesion and migration, not only in fibroblasts but also in other cell types. Peripheral blood monocytes which migrate through the interstitium come into contact with collagen I. In vitro studies have showed that collagen I markedly increases interleukin-8 expression compared to cells grown on plastic [18]. In all species, the milk proteins are specifically expressed in the mammary gland under the control of lactogenic hormones and the extracellular matrix [19]. A different integrin, α3β1, is expressed on colon carcinoma cells. Differentiation of these cells on collagen gels has been observed. Additionally, the monoclonal antibody against the β1 subunit interrupts this differentiation [20]. This suggests that the differentiated state is dependent on collagen-integrin interactions. The breast stromal fibroblasts normally deposit collagen, but this becomes excessive in the presence of an invasive tumor. There is usually an intense stromal reaction (desmoplasia) to invasive tumors which results in excessive collagen deposition and fibroblast proliferation and the significance of this stromal reaction is unclear.

The exact paracrine interactions between normal epithelial and stromal cells remain unclear and the importance of dysregulation of these interactions in can-

cer is unknown. The studies reported here focus upon the role of the extracellular matrix protein, collagen I, on aromatase activity in breast stromal cells in culture. Further experimentation evaluates the direct paracrine interactions of soluble factors produced by the stroma that can support tumor growth.

2. Materials and methods

2.1. Cell cultures

Cell cultures were maintained using a supplemented DMEM media (Gibco), without phenol red and containing 1.5× essential amino acids, 1.5× vitamin and 2× nonessential amino acids. The sterilized liquid media was prepared by the OSU Comprehensive Cancer Center by dissolving the powder into water containing sodium chloride (8.3 mM), pyruvic acid (1.25 mM) and sodium bicarbonate (17.5 mM). Cells were maintained at 37°C, 5% CO_2 and 85–95% humidity (Forma model 3052) using Corning culture flasks and plates. Cells were grown to 80% confluence and split as needed for experiments using a typsin (0.5%) EDTA mixture (Gibco). MCF-7 cells (#22-HTB; breast adenocarcinoma, ER+) were obtained from The American Type Culture Collection (ATCC) and were stored in liquid nitrogen (−196°C) until needed. The passage number for the MCF-7 cell line was monitored and cells discarded after 10 serial passages (155–165) when used in gene expression studies.

Primary breast fibroblasts were isolated from patient breast tissues obtained through the Tissue Procurement facility of the OSU Comprehensive Cancer Center. Samples (0.5 g each) were obtained from patients undergoing reduction mammoplasty or mastectomy. Tissue was stored in DMEM without serum at 4°C until used. Samples were then asceptically minced and extraneous fat and connective tissue removed. Minced tissue was incubated with 1% collagenase type III (Gibco) with shaking at 37°C for 12–15 h or until all of the pieces were digested. Samples sat for 30 min to let any undigested fragments settle. The supernatant was recovered and centrifuged at 1500 *g* for 5 min. Lipid accumulated at the top and was removed with the supernatant. Cells were resuspended in media and centrifuged for 1 min at 300–500 *g*. Under these conditions epithelial cells pellet and the resulting fibroblast enriched supernatant was plated in T-75 culture flasks in DMEM/F-12 Ham's mixture (Gibco) containing 10% fetal calf serum was added, in addition to L-glutamine (5 mM) and gentamicin (0.025%). Cultures were grown until they reached near confluence and then used in experiments. Under the conditions described, any contaminating epithelial component will not plate and will be washed away with regular media changes.

Fetal calf serum was obtained from Gibco and was used at 10% in all media unless noted. Serum was stripped of endogenous steroids by two treatments with a dextran coated charcoal mixture. A suspension (5 ml) of dextran coated charcoal (0.5% charcoal, 0.05% dextran and 0.14 M NaCl) was added to 100 ml of serum and incubated at 50°C for 30 min. This method has been shown to remove 98% of endogenous steroids from the serum [21]. Stripped serum was used in experiments where cells could not be exposed to endogenous estrogens. Collagen I coated plates were prepared in the following way. Rat tail collagen I was purchased from Collaborative Biomedical Products at a concentration of 4 mg/ml. The final collagen concentration in the gel is 1mg/ml in PBS. This concentration provides a gel of sufficient strength to allow media to be removed and added without the loosing integrity of the gel. The following mixture was used for 1 ml of collagen I mixture (85 μl 10× PBS; 210 μl 1N NaOH; 450 μl H_2O and 250 μl of collagen I). A volume of 0.8 ml of the collagen mixture was added to 6 well plates (Corning) and placed at 37°C for 30 min to allow gel to form. Cells were plated directly on top of the gel.

2.2. cDNA probes

The cDNA sequences were used as cDNA probes for Northern analysis. Plasmids for pS2 (#57136), 36B4 phophoriboprotein PO (#65917) and vimentin (#59161) were purchased from ATCC stocks. PCR primers were synthesized by Oligo's Etc (Wilsonville, OR) which would amplify regions of each of these genes to be used as cDNA probes for Northern analysis. The primer sequences for pS2 were 5′-ATC CCT GAC TCG GGG TCG CCT TTG-3′ (sense, bases 1–25) and 5′-CAA TCT GTG TTG TGA GCC GAG GCA CAG-3′ (antisense, bases 408–434). Amplification using these primers produces a 0.434 kB cDNA product for Northern analysis. The primer sequences for 36B4 were 5′-AAA CTG CTG CCT CAT ATC CG-3′ (sense, bases 306–325) and 5′-TTT CAG CAA GTG GGA AGG TG-3′ (antisense, bases 848–867). Amplification using these primers produces a 0.562 kB cDNA product for Northern analysis. The vimentin ATCC plasmid was transformed into competent DH5α cells (Gibco BRL) and plated onto LB plates with ampicillin. Positive colonies were isolated for large scale midi-preps and plasmids were isolated. Plasmids were digested with EcoR1 and the 1.1 kB fragment corresponding to 331–1431 bp was isolated using the Quiagen band prep system. Radiolabeled cDNA probes were prepared by the random priming method

(Rad Prime Labeling Kit, Gibco BRL) using 50 μCi of [α-^{32}P]-dCTP.

2.3. Aromatase activity in primary fibroblasts

Fibroblasts were grown on either collagen I gels or plastic and cells were plated at a concentration of 5×10^5 cells/well in 6-well plates. Aromatase activity was determined by measuring the release of tritiated water from [1β-^{3}H]-androst-4-ene-3,17-dione following published procedures [22,23]. Cells were given fresh media (3 ml) and were incubated with 2 μCi/well of [1β-^{3}H]-androst-4-ene-3,17-dione (80 nM total androgen concentration) with an incubation time of 12 h. Separate blank samples were used for the plastic and collagen wells which had no cells plated. After 12 h, the media was removed and extracted with chloroform three times. Aliquots (500 μl) of the media were transferred into 12 × 75 mm culture tubes, treated with 1% dextran coated charcoal (500 μl) and centrifuged at 2600 g for 10 min. Aliquots (500 μl) of the aqueous solutions were removed, added to scintillation cocktail and radioactivity measured on a liquid scintillation counter.

2.4. Thymidine incorporation

The measurement of tritiated thymidine incorporation allows for a correlation to growth of cells. The incorporation of thymidine was carried out using primary breast fibroblasts which were grown on either plastic or collagen I. Cells were plated at 5×10^5 cells/well and separate flasks were used for each of the seven days. Thymidine (1 μC/well) was added to each plate in triplicate for each time point. Incubation was performed for eight hours at 37°C. After the incubation, cells were washed two times and then lifted using either trypsin:EDTA or collagenase type III depending on which type of plate was used. Cells were centrifuged and washed twice using PBS. The supernatent was decanted and 1 ml of 5% TCA solution was added and left at 4°C overnight. The mixture was centrifuged at 3000 × g for 10 min at 4°C and the supernatant decanted. A 1 ml mixture of 0.1N NaOH and 1% Triton N101 was added to each pellet and vortexed vigorously. An aliquot of 270 μl of mixture was added to 5 ml of cocktail which had been acidified with 31 μl of 1N HCl. Amount of radioactive thymidine incorporated into the cellular DNA was counted using a scintillation counter.

2.5. RNA isolation and northern analysis

RNA isolation was performed using a modification of the method described by Chomczynski [19]. MCF-7 cells in 6-well plates were washed with phosphate buffered saline and 500 l of guanidine isothyanate (3 M) solution was added to each well. The lysate was sheared with a 26 gauge needle and transferred to a phase lock gel tube (5′-3′, Boulder, CO). Fifty microliters of 3 M sodium acetate (pH 5.0), 500 μl of phenol (pH 5.0) and 200 μ1 of chloroform:isoamyl (28:1) were added separately. The tubes were centrifuged at 12,000 *g* for 2 min to separate the phases and the extraction was repeated. The supernatant was combined with an equal volume of isopropanol and allowed to precipitate at −20°C for 2 to 16 h. The precipitate was centrifuged at 14,000 g for 30 min and the resulting pellet washed twice with 70% ethanol and once with 95% ethanol. The RNA was reconstituted in RNAse-free water and absorbance measured at 260 nm to quantify total RNA.

Ten microgram samples of total RNA were run on 1.2% agarose gel at 90 V for 90 min and transferred to Nytran Plus membranes (Schleicher and Schuell, Keene, NH). The membranes were baked for 1 h at 80°C, prehybridized with a solution containing 50% formamide, 5× Denhardts solution, 5× SSPE, 2% SDS and salmon sperm DNA (100 μg/ml) for 2 h at 42°C. Membranes were hybridized with randomly labeled (RadPrime kit, GIBCO BRL) [α-^{32}P]-dCTP pS2 (2×10^6 cpm/ml) and 36B4 (1×10^6 cpm/ml) cDNA probes in a solution containing 50% formamide, 5× Denhardts solution, 5× SSPE, 1% SDS and salmon sperm DNA (100 μg/ml) for 16–20 h at 42°C. Final wash was done at 65°C with 0.1× SSPE and 1% SDS. Membranes were scanned with a radioanalytical imaging detector (Ambis Inc., San Diego, CA) and the signals quantified according to pS2 to 36B4 ratios to control for RNA loading discrepancies. All experiments were carried out in duplicate flasks and replicated; results represent the average of four data points. Statistical analysis was performed using the Student t-test (unpaired).

3. Results

3.1. Aromatase activity of primary fibroblasts

Primary normal stromal fibroblasts cells obtained from breast tissue samples from twelve female patients of varying age (16–80 yr) were evaluated for aromatase activity when grown under different culturing conditions. The effect of the extracellular matrix protein, collagen I, on aromatase activity was investigated. Aromatase plays a critical role in regulating levels of the estrogen in the breast and collagen I is the major ECM protein in the breast stroma. Therefore, investigations on the effects of collagen I on the regulation of this enzyme is warranted. Although several studies have examined fibroblast aromatase activity, no study

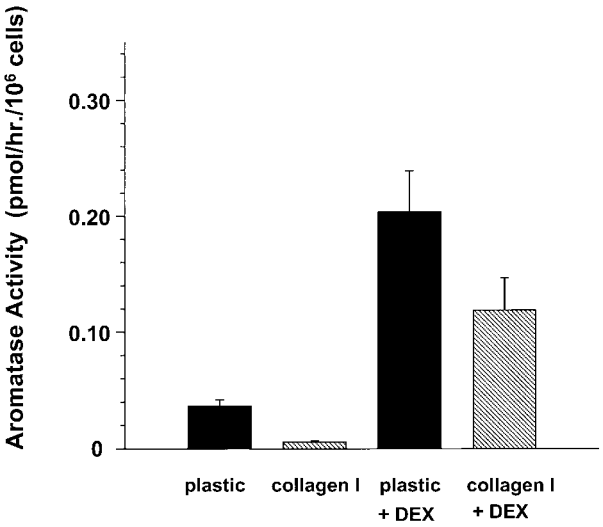

Fig. 1. Aromatase activity in breast stromal cells cultured on varying support matrix. Cells were cultured on plastic or on collagen I gels in the absence or presence of 100 nM dexamethasone. $N = 10$ patients.

has used an appropriate substratum which mimics in vivo conditions.

Constitutive aromatase activity was measured in cultured primary stromal fibroblasts (Fig. 1). Additionally, the ability of these cells to respond to dexamethasone was determined. Normal fibroblasts utilize promoter I.4 which has a glucocorticoid responsive element located upstream of the untranslated exon I.4, making them responsive to treatment with 100 nM dexamethasone. Constitutive aromatase activity was found to be lower when cells were grown on a collagen gel for 4–7 days (6.0 fold lower) using DMEM/F12 containing 10% dextran coated charcoal stripped serum. Fibroblasts grown on collagen I also appeared to be significantly more responsive to stimulation by

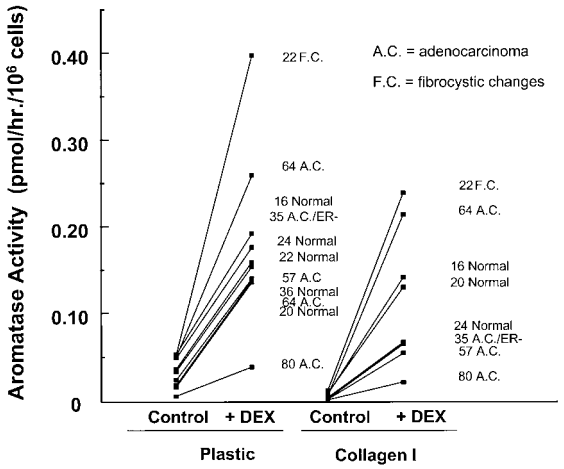

Fig. 2. Comparison of aromatase activity in cultured breast stromal cells among individual patients. Cells were cultured on plastic or on collagen I gels in the absence or presence of 100 nM dexamethasone.

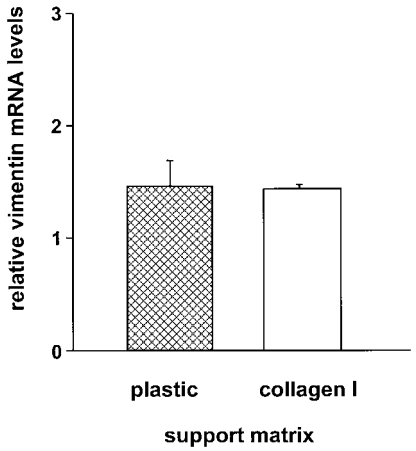

Fig. 3. Relative expression of vimentin mRNA in breast stromal cells cultured on varying support matrix. Cells were cultured on plastic or on collagen I gels. $N = 5$ patients.

100 nM dexamethasone (plastic: 7.0 fold induction, collagen: 33.2 fold induction) when pretreated for 12 h prior to measurement of aromatase activity. Thus, culturing primary fibroblasts on a collagen I matrix resulted in a decrease in constitutive aromatase activity. This decrease corresponded to a six-fold reduction in activity compared to cells cultured on plastic support. These values were corrected for the number of cells so this reduction is not a reflection of differences in the number of cells.

Significant variability of aromatase levels in response to dexamethasone induction existed in the stomal cell culture of this patient group. Figure 2 presents this data as individual patients rather than as a group. Varying levels of induction have also been reported in the literature and loosely correlate with age [24,25].

3.2. Expression of vimentin in fibroblasts grown on collagen

The expression of vimentin mRNA was measured by Northern analysis in order to evaluate the effect of collagen I on fibroblasts. Vimentin is an intermediate filament protein which is expressed by fibroblasts and cells of mesenchymal origin. Fibroblasts were cultured for four days and the levels of steady state mRNA were measured using Northern analysis and a cDNA probe specific to vimentin. No significant difference in the expression of vimentin was observed when fibroblasts were grown on collagen I compared to plastic (Fig. 3).

3.3. Thymidine incorporation

The relative growth rates of fibroblasts grown on plastic and collagen I were evaluated by monitoring the incorporation of radiolabeled thymidine.

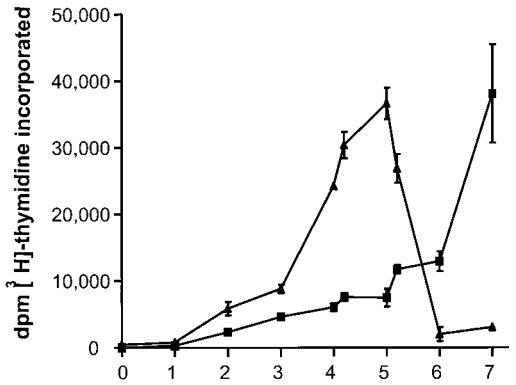

Fig. 4. Thymidine incorporation in breast stromal cells cultured on varying support matrix. Cells were cultured on plastic (▲) or on collagen I gels (■). $N = 6$ wells.

Fibroblasts were grown on plastic or collagen I for 1 to 7 days. Radiolabelled thymidine was added to separate plates every day. The incorporation of thymidine can be related to the growth rates of these cells (Fig. 4). The fibroblasts which were grown on collagen I showed a significant reduction in the incorporation of thymidine from day 1 to day 5. The cells that were grown on plastic had a faster growth rate and reached confluence at around day 5. These cells grown on plastic halted growth at confluence due to contact inhibition, resulting in the drastic decrease in tritiated thymidine incorporation after 5 days for the cells grown on plastic support. Thus, breast fibroblast cells exhibited a delay in growth of three days when cultured on collagen I support matrix.

3.4. Conditioned media experiments

In order to investigate further the potential importance of stromal aromatase activity in breast tissue, experiments were performed with MCF-7 cells using conditioned media from fibroblasts cultured under a variety of conditions. Soluble factors secreted by the fibroblasts into the media would then be able to interact with the epithelial MCF-7 cells. Additionally, conversion of androgen precursor administered to the stromal cells to estrogen would elicit an estrogen-mediated response in the epithelial cells if estrogen levels were of sufficient magnitude.

MCF-7 cells are known to express a small trefoil protein, known as pS2. This is a small 84 residue protein, containing at its amino terminus a signal peptide characteristic of secreted proteins, which was isolated from a human breast carcinoma cell line. Transcriptional activation of the pS2 gene is a primary response to estrogens in the MCF-7 cell line [26,27]. The proximal 5′ flanking region of the pS2 gene contains a transcriptional enhancer estrogen responsive element, a 13 base pair imperfect palindromic sequence.

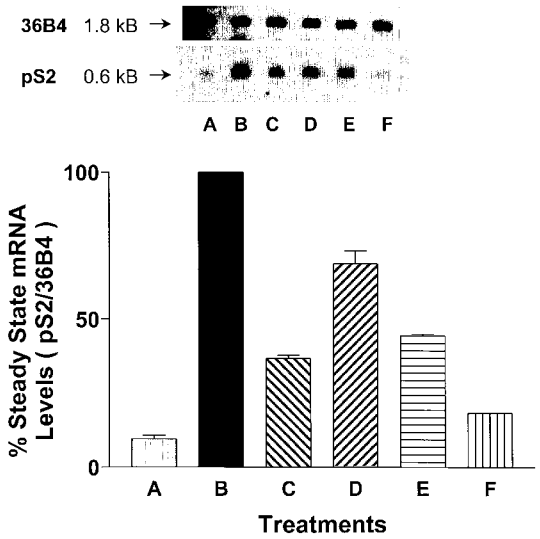

Fig. 5. Northern analysis of the effect of fibroblast-derived conditioned media on pS2 mRNA expression in MCF-7 cells. (A) Control media. (B) Media containing 10 nM estradiol. (C) Conditioned media from untreated breast stromal cells. (D) Conditioned media from breast stromal cells treated with 100 nM testosterone. (E) Conditioned media from breast stromal cells treated with 100 nM testosterone and 100 nM dexamethasone. (F) Conditioned media from breast stromal cells treated with 100 nM testosterone and 100 nM a potent aromatase inhibitor (7α-APTADD). Each bar is the mean of four samples, with the err bars representing the standard err of the mean (S.E.M.).

Cells which are estrogen deprived for 48 h show a low level of this message and it becomes markedly stimulated by the addition of estradiol. The increase in pS2 message is rapid, with an increase in mRNA expression after 12 h of treatment and a maximum after 48 h.

If the fibroblasts were producing estrogens from provided precursor, then the estrogen responsive MCF-7 cells would respond by an increase in pS2 gene expression. The pS2 mRNA levels are an indirect measure of the estrogen produced at levels that are able to elicit a response in a neighboring cell.

Conditioned media from fibroblasts supplemented with 100 nM testosterone as the provided androgen or with vehicle only was administered to MCF-7 cells which had been estrogen deprived for 48 h. Other fibroblast cultures received testosterone, dexamethasone and/or 7α-APTADD, which would inhibit conversion of testosterone. Following incubations, RNA was isolated and mRNA for pS2 and 36B4 were analyzed by Northern analysis (Fig. 5). The control flasks, which were deprived of estrogen for the course of the experiment, showed a pS2 expression which was 9.6% of the maximal response of pS2 (normalized to 100% for estradiol at 10 nM). Conditioned media from fibroblasts showed an increase in pS2 expression corresponding to 36.4% of the value for 10 nM estradiol.

The addition of 100 nM testosterone resulted in a

further increase in pS2 expression over the conditioned media alone, producing 68.8% of control. The effect of adding dexamethasone is dependent on the length of treatment. In short term treatments (i.e. less than 24 h), dexamethasone increases aromatase activity in fibroblasts, as was shown earlier in this study. However, a report evaluating the result of longer dexamethasone treatment determined that long-term treatment of greater than 36 h decreases aromatase activity and decreases glucocorticoid receptor levels [28]. In this study, dexamethasone was added at the beginning of the 3-day treatment and resulted in a reduction of pS2 expression at 44.3%. There was no statistical difference in the pS2 expression between conditioned media alone and the testosterone plus dexamethasone treatment. The addition of 7α-APTADD, a potent enzyme-activated irreversible aromatase inhibitor [29], results in a reduction in pS2 expression to 18.3%. These observations suggest that testosterone is being converted into estradiol by aromatase present in the stromal cells.

An estrogen-independent increase in pS2 expression was observed in MCF-7 cells given conditioned media alone (Fig. 5). Factors produced by the fibroblasts that cause increased pS2 expression in these experiments are not known at this time. Reports in the literature suggest that cytokine IL-6 and growth factors TGF-α and insulin-like growth factor IGF-1, when added to flasks of astrocytes and MCF-7 cells, were able to induce a pS2 increase [30,31]. Interestingly, normal fibroblasts have been shown to express large amounts of IGF-1 [32], while fibroblasts isolated directly from the tumor seem to express large amounts of IGF-2 and no IGF-1 [33]. The fibroblasts themselves do not become genetically altered in breast tumors but the secreted factors are markedly affected by the presence of the tumor. IGF-2 has not been shown to increase pS2 expression.

4. Conclusions

A collagen I matrix has a measurable affect on the aromatase activity of primary breast fibroblasts. The levels of aromatase activity were significantly increased by a short-term treatment of 100 nM dexamethasone, demonstrating that these primary fibroblast grown on a collagen I matrix retain their hormone responsive state. No alteration in the intermediate filament protein vimentin in primary fibroblasts cells grown on either plastic or collagen I support was observed. Collagen I reduced incorporation of radiolabelled thymidine in cultured fibroblasts compared to fibroblasts on plastic support and culturing cells on collagen I may allow for a more differentiated phenotype as a consequence of slowed growth. This data supports the specific interaction of the fibroblast VLA-2 integrin receptor with a collagen I matrix, possibly resulting in chromatin alterations which affect gene transcription.

Historically, cell culture systems have been optimized for maximal growth. Since growth and differentiation are two mutually exclusive cellular functions, a cell culture system that offers maintenance of cells rather than supporting maximal proliferation may be more representative of in vivo conditions. The rationale of growing cells on a collagen I substratum was that this would provide a more relevant model for conditions in the breast. It is critical that measurement of gene expression and enzyme function be carried out under circumstances seen in the normal breast in order to determine the importance of the stromal fibroblasts to the growing tumor.

In an effort to examine paracrine interactions between stromal and epithelial cell types, investigations using conditioned media from fibroblast cultures were performed. These experiments demonstrated that secreted products from fibroblasts can induce pS2 expression in MCF-7 cells. First, estrogen-independent factor(s) are released into the media and are capable of stimulating pS2 expression. Second, testosterone administration to fibroblasts results in the production of estradiol by aromatase into the media in sufficient concentrations to elicit an increase in pS2 expression when the conditioned media is administered to MCF-7 cells. The addition of an aromatase inhibitor resulted in a complete suppression of fibroblast-derived estrogens and significant decrease in pS2 expression.

These experiments highlight the need for evaluation of gene expression in cell culture systems that accurately reflect the tissue environment. In the discussion of the importance of the local production of estrogens in the breast, it seems necessary to take into consideration the relative aromatase activity of the two major cell types. The fibroblasts, based on previous cell culture data, were thought to have the majority of the estrogen producing capabilities. If, however, in vivo they have lower levels constitutively, their role may not be singularly paramount. Furthermore, the potential for high aromatase activity in breast stromal cells may more critically depend on tumor-derived factors.

Acknowledgements

This work was supported in part by grants R01 CA73698, T32 CA09498, R21 CA66193 and P30 CA16058 from the National Cancer Institute, National Institutes of Health.

References

[1] V.H.T. James, J.M. McNeill, L.C. Lai, C.J. Newton, H. Braunsberg, M. Ghilchik, M.J. Reed, Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies, Steroids 50 (1987) 269–279.

[2] W.R. Miller, P. Mullen, P. Sourdaine, C. Watson, J.M. Dixon, J. Telford, Regulation of aromatase activity within the breast, J. Steroid Biochem. Mol. Biol. 61 (1997) 193–202.

[3] M.J. Reed, L. Topping, N.G. Coldham, A. Purohit, M.W. Ghilchik, V.H.T. James, Interleukin-I and interleukin-6 in breast cyst fluid: their role in regulating aromatase activity in breast cancer cells, J. Steroid Biochem. Mol. Biol. 44 (1993) 589–596.

[4] M.J. Reed, The role of aromatase in breast tumors, Breast Canc. Res. Treat. 30 (1994) 7–17.

[5] S.E. Bulum, T.M. Price, M.S. Mahendroo, J. Aitken, E.R. Simpson, A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcriptase, J. Clin. Endocrinol. Metab. 77 (1993) 1622–1628.

[6] C.E. Sekeris, Hormonal steroids act as tumor promoters by modulating oncogene expression, J. Cancer Res. Clin. Oncol. 117 (1991) 96–101.

[7] M.W. Hashimoto, O. Nikaido, N. Kobayashi, C.C. Chang, J.E. Trosko, T. Mori, A comparison of the propensity for gene amplification between near tetraploid and near diploid V79 clones resistant to methotrexate, Carcinogenesis 17 (1996) 389–394.

[8] C.Q. Lin, M.J. Bissell, Multi-faceted regulation of cell differentiation by extracellular matrix, FASEB J. 7 (1993) 737–743.

[9] A. Lochter, M.J. Bissell, Involvement of extracellular matrix constituents in breast cancer, Semin. Cancer Biol. 6 (1995) 165–173.

[10] P.L. Jones, C. Schmidhauser, M.J. Bissell, Regulation of gene expression and cell function by extracellular matrix, Crit. Rev. Eukaryotic Gene Expr. 3 (1993) 137–154.

[11] A.R. Howlett, M.J. Bissell, The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium, Epithelial Cell Biol. 2 (1993) 79–89.

[12] E.W. Thompson, M. Yu, J. Bueno, et al., Collagen induced MMP-2 activation in human breast cancer, Breast Cancer Res. Treat. 31 (1994) 357–370.

[13] C.Q. Lin, P.J. Dempsey, R.J. Coffey, M.J. Bissell, Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-(in mouse mammary epithelial cells: studies in culture and in transgenic mice. , J. Cell Biol. 129 (1995) 1115–1126.

[14] L.M. Houdebine, J. Djiane, Effects of lysosomatotropic agents and of microfilament and microtubule disrupting agents on the activation of the beta casein gene expression by prolactin in the mammary gland, Mol. Cell Endocrinol. 17 (1980) 1–6.

[15] D.A. Jackson, P.R. Cook, Replication occurs at a nucleoskeleton, EMBO J. 5 (1986) 1403–1410.

[16] D.A. Jackson, P.R. Cook, Transcription occurs at a nucleoskeleton, EMBO J. 4 (1985) 919–925.

[17] P.L. Jones, N. Boudreau, C.A. Myers, H.P. Erickson, M.J. Bissell, Tenascin-C inhibits extracellular matrix-dependent gene expression in mammary epithelial cells. Localization of active regions using recombinant tenascin fragments, J. Cell Sci. 108 (1995) 519–527.

[18] L.A. Heinel, D. Singleton, M. Miller, M.B. Frewin, P.W. Gudewicz, Monocyte adherence to the subepithelial basement membrane increases interleukin-8 gene expression and antigen release, Inflammation 19 (1995) 517–527.

[19] M. Bayat-Sarmadi, C. Puissant, L.M. Houdebine, The effects of various kinase and phosphatase inhibitors on the transmission of prolactin and extracellular matrix signals to rabbit alpha S1-casein and transferrin genes, Int. J. Biochem. Cell Biol. 27 (1995) 707–718.

[20] L.A. Liotta, U. Wewer, N.C. Rao, et al., Biochemical mechanism of tumor invasion and metastasis, Anticancer Drug Des. 2 (1987) 195–202.

[21] R.L. Eckert, B.S. Katzenellenbogen, Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells, Cancer Res. 42 (1982) 139–144.

[22] R.W. Brueggemeier, N.E. Katlic, Effects of an enzyme-activated irreversible aromatase inhibitor on human carcinoma cells in culture, Cancer Res. 50 (1990) 3652–3656.

[23] W.E. Burak, A.L. Quinn, W.B. Farrar, R.W. Brueggemeier, Androgens influence estrogen induced responses in human breast carcinoma cells through cytochrome P450 aromatase, Breast Cancer Treat. Res. 44 (1997) 57–64.

[24] G.D. Berkovitz, S. Chen, C.J. Migeon, M.A. Levine, Induction and superinduction of messenger ribonucleic acid specific for aromatase cytochrome P-450 in cultured human skin fibroblasts, J. Clin. Endocrinol. Metab. 74 (1992) 629–634.

[25] G.D. Berkovitz, K. Carter, C. Migeon, T. Brown, Down-regulation of the glucocorticoid receptor by dexamethasone in cultured human skin fibroblasts: implications for the regulation of aromatase activity, J. Clin. Endocrinol. Metab. 66 (1988) 1029–1036.

[26] M.-C. Rio, P. Chambon, The pS2 gene, mRNA and Protein, a potential marker for human breast cancer, Cancer Cells, Monthly Rev. 2 (1990) 269–274.

[27] M.C. Rio, J.P. Bellocq, B. Gairard, et al., Specific Expression of pS2 gene in a subclass of breast cancers in comparison with expression of the estrogen and progesterone receptors and ERBB2, Proc. Natl. Acad. Sci. 84 (1987) 9243–9247.

[28] G.D. Berkovitz, S. Chen, C.J. Migeon, M.A. Levine, Induction and superinduction of messenger ribonucleic acid specific for aromatase cytochrome P-450 in cultured human skin fibroblasts, J. Clin. Endocrinol. Metab. 74 (1992) 629–634.

[29] C.E. Snider, R.W. Brueggemeier, : Potent enzyme-activated inhibition of aromatase by a 7α-substituted C_{19} steroid, J. Biol. Chem. 262 (1987) 8685–8689.

[30] D. Chalbos, A. Philips, F. Galtier, H. Rochefort, Synthetic antiestrogens modulate induction of pS2 and cathepsin-D messenger ribonucleic acid by growth factors and adenosine 3′,5′-monophosphate in MCF7 cells, Endocrinol. 133 (1993) 571–576.

[31] M. Hirota, H. Awatsuji, Y. Furukawa, K. Hayashi, Cytokine regulation of PS2 gene expression in mouse astrocytes, Biochem. Mol. Biol. Int. 33 (1994) 515–520.

[32] A. Manni, B. Badger, L. Wei, et al., Hormonal regulation of insulin-like growth factor II and insulin-like growth factor binding protein expression by breast cancer cells in vivo: evidence for stromal epithelial interactions, Cancer Res. 54 (1994) 2934–2942.

[33] M.J. Ellis, C. Singer, A. Hornby, A. Rasmussen, K.J. Cullen, Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer, Breast Cancer Res. Treat. 31 (1994) 249–261.