Mammary Fibroblast Influence on Normal Mouse Mammary Epithelial Cell Responses to Estrogen in Vitro

Sandra Z. Haslam
Anatomy Department, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Estrogen-dependent stimulation of progesterone receptor (PgR) concentration or cell proliferation of normal mammary epithelial cells in vitro has been shown to be associated with the presence of mammary fibroblasts. To investigate further the nature of fibroblast influence on epithelial cells, Percoll-purified epithelial cells from collagenase-dissociated mammary glands of mid-pregnant BALB/c mice were co-cultured with mammary fibroblasts that were either untreated, irradiated, or glutaraldehyde-killed or with fibroblast-conditioned medium. Epithelial cells were then assayed for either estrogen-dependent stimulation of PgR by measuring specific [3H]R5020 binding or for estrogen-dependent stimulation of DNA synthesis by [3H]thymidine autoradiography. The results demonstrate that stimulation of PgR does not require the presence of live fibroblasts; either glutaraldehyde-killed fibroblasts or conditioned medium was effective. Pretreatment of culture dishes with type I collagen was equally effective, indicating that fibroblasts may promote the PgR response via a substratum effect. In distinct contrast, estrogen-dependent stimulation of DNA synthesis occurred only when live fibroblasts were present in high numbers and/or in direct contact with epithelial cells. Furthermore, under these latter conditions, epithelial cells also promoted estrogen-dependent stimulation of fibroblast DNA synthesis. Differences in both epithelial and fibroblast cell morphologies were also observed under co-culture conditions, which suggested that cell-cell communication or another interactive phenomenon takes place and is bidirectional.

Thus there appear to be at least two different mechanisms by which fibroblasts can influence two specific responses of epithelial cells to estrogen. The present results demonstrate that the specific nature of epithelial-stromal interactions can determine and modulate epithelial cell responses to estrogen and may reflect in vivo regulatory processes affecting normal and neoplastic mammary cells.

INTRODUCTION

The mammary gland is distinct from many other epithelial organs in that its major morphogenesis occurs postnataally, during pregnancy, and is hormonally regulated (1). In rodents estrogen is required for mammary cell proliferation and has been shown to increase mammary DNA synthesis (2); estrogen also acts to increase PgR2 concentration (3). Estrogen has also been strongly implicated in the control of human mammary tumor cell proliferation (4). Several lines of evidence indicate that the morphogenic processes which occur during pregnancy are similar to those operative during fetal development of the organ and are dependent upon epithelial-stromal interactions (5, 6). At present little is known about epithelial-stromal interactions and hormonally regulated growth and function in the adult mammary gland. Advancing our knowledge in this area may provide important insights into mechanisms of normal growth regulation and its aberrations in cancer.

Recently we have shown that estrogen increases PgR concentration in normal mouse mammary epithelial cells in vitro (7). However, this appears to be the case only in mixed cultures which contain both mammary epithelial cells and mammary fibroblasts. In contrast, cultures highly enriched for epithelial cells do not maintain their responsiveness to estrogen, despite the presence of estrogen receptors. The estrogen effect appears to be specific for the epithelial cell population of mixed cultures, since fibroblasts cultured alone do not contain PgR even when treated with estrogen. Mammary fibroblasts have also been shown to be associated with estrogen-dependent cell proliferation of mammary epithelial cells in vitro (8).

The favorable effect of fibroblasts on epithelial cells could be due to supplementation of the medium with necessary undefined factors. Conditioned medium from cultured mouse mammary fibroblasts has been reported previously to stimulate normal mouse mammary epithelial cell proliferation in vitro, and it has been proposed that a fibroblast-derived growth factor(s) is responsible for the stimulatory effect of the conditioned medium (9). On the other hand the physical nature and chemical composition of the culture substratum are also known to be important for normal mammary epithelial cell function in vitro (10). Fibroblasts might function to improve the culture substratum. Additionally, it has been proposed that direct contact between epithelial cells and fibroblasts is required to promote estrogen-dependent cell proliferation (8).

The mechanism(s) by which mammary fibroblasts influence epithelial cell responses to estrogen has not been delineated. In this report we have investigated further the nature of mammary fibroblast effect on estrogenic regulation of PgR and estrogen-dependent DNA synthesis of normal mouse mammary epithelial cells in vitro.

MATERIALS AND METHODS

Reagents. Collagenase III and Pronase were from Worthington Biochemical Corp. (Freehold, NJ) and Calbiochem (LaJolla, CA), respectively. All culture media ingredients were from Grand Island Biological Co. (Grand Island, NY). R5020 (87 Ci/mmol) and radioinert R5020 were from New England Nuclear Corp. (Boston, MA). All other hormones were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Cell Culture. BALB/c mice from our own colony, 2–5 months old and...
14–18 days pregnant, were the source of mammary tissue. Tissue was dissociated with 0.1% collagenase, and epithelial cells and fibroblasts were separated by differential centrifugation and Percoll gradients as described (7). Epithelial cells or fibroblasts were plated separately in 2-

mm² plastic multiwell dishes (Nunc) at a density of 2 × 10⁴ viable cells/cm² in Medium 199 containing 5% charcoal-treated FCS, insulin (0.006 g/ml), ovine prolactin (1 mg/ml), and 10⁻⁶ M cortisol. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂ in air; medium was changed every 2 days. The relative purity of epithelial (97%) or fibroblast (100%) cultures has been established previously (7).

**Fibroblast Treatments and Co-Culture Conditions.** Fibroblasts were grown to confluence in culture medium; control fibroblasts received no further treatment. Irradiated fibroblasts received 6000 rads using a 60Co irradiator 24 h prior to co-culture with epithelial cells.

GK fibroblasts were prepared as described (11). Confluent fibroblasts were incubated with 3% glutaraldehyde in Ca²⁺, Mg²⁺-free phosphate-buffered saline (0.8% NaCl, 2.7 mM KCl, 7.6 mM Na₂HPO₄, 5.5 mM KH₂PO₄, pH 7.4) for 30 min at room temperature. The cells were then immediately washed 3 times in phosphate-buffered saline followed by successive washing every hour for 8 h and then kept in Medium 199 at 37°C for 24 h prior to co-culture with epithelial cells.

To obtain CM, fibroblast cultures were incubated for 24 h in Medium 199 without FCS or hormones. The CM was passed through a Millipore filter (pore size, 0.5 μm) and stored at −20°C until used (9). Prior to use CM was diluted 50% with fresh Medium 199 and had the same final concentration of FCS and hormones as the regular medium.

For co-culture experiments epithelial cells were plated onto control or treated confluent fibroblast monolayers at a density of 2 × 10⁴ cells/cm². In experiments in which epithelial cells were co-cultured with fibroblasts, each cell type was plated at 2 × 10⁵ cells/cm².

Rat tail collagen prepared as described (10) was the source of type I collagen. Culture dishes were pretreated by incubating with 0.25 ml soluble collagen for 24 h at room temperature; the dishes were washed with Medium 199 prior to plating.

**Progestosterone Receptor Assay.** Intact epithelial cells plated alone or co-cultured with fibroblasts were grown for the indicated length of time and then were assayed for specific [³H]R5020 binding while still attached to culture dishes as described (7). Cells were incubated with [³H]R5020 (1–12 nM) with or without a 100-fold excess radioinert R5020. To correct for R5020 binding to glucocorticoid binding sites, total binding was measured in the presence of 100-fold excess radioinert dexamethasone (12). All [³H]R5020 binding data were analyzed by the method of Scatchard (13).

DNA was quantitated using parallel cultures which had not been assayed for hormone binding as described (7) according to the method of Ceriotti (14) using calf thymus DNA as the standard. To determine epithelial DNA content under co-culture conditions, DNA content was also determined in parallel cultures which contained only fibroblasts which had received identical handling. The DNA content of epithelial cells of each type was counted.

**Measurement of DNA Synthesis.** Cells were plated, in parallel, on glass coverslips, and autoradiography was as described (8) using [³H]-thymidine (6.7 Ci/mmol) (ICN Radiochemicals, Irvine, CA). Labeling was for 1 h. Duplicate coverslips were analyzed for each experimental point. Labeled nuclei were counted in 20 random high-power (400x) microscope fields containing epithelial cells or fibroblasts. A minimum of 1000 cells of each type was counted.

**Statistical Analysis.** All data are expressed as the mean ± SE and were analyzed for significance using Student’s t-test or analysis of variance where appropriate.

**RESULTS**

**The Nature of Fibroblast Influence on Estrogen-dependent PgR Regulation.** Confluent monolayers of mammary fibroblasts were left untreated (control), rendered proliferatively dead by irradiation, or killed by glutaraldehyde fixation (GK). Freshly dissociated mammary epithelial cells were plated onto the control or treated fibroblasts or alternatively plated on plastic and cultured with or without CM. When epithelial cells reached confluence (5–7 days later) the cultures were changed to medium with or without 20 nM 17β-estradiol; 3 days later the cultures were assayed for specific [³H]R5020 binding (7). The results presented in Chart 1 demonstrate that in all instances, co-culture with fibroblasts resulted in an estrogen-dependent stimulation of PgR (Kₚ = 1.59 ± 0.08 × 10⁻⁹ M, n = 39). The increases in PgR concentration ranged from 1.4- to 2-fold with irradiated fibroblasts or GK fibroblasts, respectively. It is also noteworthy that under all co-culture conditions higher basal levels of PgR were maintained in the absence of 17β-estradiol than were maintained in epithelial cells cultured alone. Direct contact between epithelial cells and fibroblasts does not appear to be required, since a comparable 1.6-fold stimulation of PgR was obtained with CM. No greater increase in PgR was obtained if epithelial cells were cultured with GK fibroblasts plus CM.

Type I collagen, an extracellular product of fibroblasts, has been shown to have beneficial substratum effects for mammary epithelium (10). When epithelial cells were plated in culture dishes which had been pretreated with soluble type I collagen (Chart 1), treatment with 17β-estradiol resulted in a 1.8-fold stimulation of PgR concentration.
The Nature of Fibroblast Influence on Estrogen-dependent Epithelial Cell DNA Synthesis. To determine the nature of fibroblast influence on epithelial cell proliferation, DNA synthesis was measured in parallel co-cultures, autoradiographically using [3H]thymidine. In these experiments epithelial cells were plated with or without 20 nM 17β-estradiol and LI were determined 1 or 5 days later; the data are shown in Chart 2. When epithelial cells were grown alone, 17β-estradiol had no stimulatory effect on DNA synthesis at either time point. In contrast co-culture with control mammary fibroblasts resulted in a significant increase in epithelial cell LI in the presence of 17β-estradiol; a 1.9- and 1.5-fold increase in LI were seen at 1 and 5 days, respectively. Fibroblasts themselves do not appear to be stimulatory in the absence of 17β-estradiol, since LI were not significantly different for epithelial cells grown alone or co-cultured with control fibroblasts. Co-culture with irradiated fibroblasts also resulted in a significant 1.5-fold 17β-estradiol-dependent increase in LI on day 1; however after 5 days of culture a stimulatory effect of 17β-estradiol was not observed.

When epithelial cells were co-cultured with GK fibroblasts, 17β-estradiol did not cause an increase in LI. In fact, the LI at both time points were significantly lower than the LI of epithelial cells grown alone. It is possible that this inhibition of DNA synthesis was due to a cytotoxic effect of GK-fibroblasts. Indirect evidence against a cytotoxic effect is provided by the unimpaired ability of these epithelial cells to respond to estrogenic stimulation of PgR (Chart 1).

Epithelial cells grown in CM also did not exhibit an increase in LI in the response to 17β-estradiol at either time point tested. It is possible that estrogen may be required for the production of the putative fibroblast-derived growth factor(s), and this could explain the lack of effect observed with CM. To test this possibility we obtained CM from fibroblasts grown with 20 nM 17β-estradiol. Prior to its use in epithelial cultures the CM was charcoal treated to remove the added estrogen. This conditioned medium also was not effective in increasing the epithelial cell LI (Chart 2).

An overall comparison among day 1 and 5 LI revealed the following. On day 1, co-culture with untreated or irradiated fibroblasts in the presence of 17β-estradiol resulted in a significant increase in LI compared to all other experimental groups. On day 5, co-culture with untreated fibroblasts also resulted in a significantly higher LI when estrogen was present. However this LI was not significantly higher than LI of epithelial cells cultured alone, co-cultured with irradiated fibroblasts or with CM in the presence or absence of estrogen. The reason for the significant increase in LI of epithelial cells co-cultured with untreated fibroblasts plus estrogen appears to be due to the lower LI observed in the control group without estrogen. Thus under these conditions, at 5 days of culture, when epithelial cells are near confluent, the presence of live fibroblasts may be inhibitory for epithelial cell proliferation if no estrogen is present.

Since epithelial cell LI was increased by estrogen upon coculture with control or irradiated fibroblasts but was not increased by CM, this suggests that close contact between epithelial cells and fibroblasts may be required. To determine if this is the case, epithelial cells were co-plated with fibroblasts. After 1 day of culture little direct contact between the two cell types was visible (Fig. 1d). At this time point no increase in LI index was observed in 17β-estradiol-treated cultures (Chart 2). In fact the LI was significantly lower than that observed for epithelial cells grown alone. However after 5 days of culture, the cells are confluent, and the epithelial cells are extensively in contact with fibroblasts (Fig. 2d); at this time 17β-estradiol-treated cultures exhibited a 1.7-fold increase in LI (Chart 2).

Epithelial Cell Effect on Fibroblast DNA Synthesis. Estrogen may stimulate DNA synthesis of mammary stromal cells in vivo (15). It was of interest to determine whether mammary fibroblasts exhibit a similar response to 17β-estradiol in vitro. Fibroblasts were plated with or without 20 nM 17β-estradiol and LI determined 1, 3, or 5 days later. No increase in LI was observed as a result of 17β-estradiol treatment (Chart 3). However if fibroblasts are co-plated with epithelial cells, treatment with 17β-estradiol results in an increased fibroblast LI at 3 and 5 days of culture (Chart 3). No stimulatory effect of 17β-estradiol was observed after only 1 day of culture; this suggests that contact with epithelial cells may also be required for the fibroblast response. These results also importantly indicate that fibroblast-epithelial cell influences appear to be bidirectional.

Appearance of Cultured Cells. Epithelial cells plated on tissue culture plastic or glass coverslips grew out from small clumps of cells to form colonies with typical polygonal epithelial cell morphology. Initially (1–3 days) the colony edges had rounded contours (Fig. 1a). By days 5–7 the colonies coalesced and the culture became confluent (Fig. 2a). By this time the epithelial
Epithelial cells cultured alone or co-cultured as indicated were counted with the aid of a microscope (400x) using an ocular grid, after 1 or 5 days of culture. In all cases the cells were cultured without estrogen and were counted in regions of the culture surface where they covered the entire grid area.

Table 1  
Effect of culture condition on epithelial cell size

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Mean no. of cells per area of culture surface</th>
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<tbody>
<tr>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Epithelial cells co-cultured with:</td>
<td></td>
</tr>
<tr>
<td>Untreated fibroblasts</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>Irradiated fibroblasts</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>GK fibroblasts</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td>53 ± 3</td>
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*P < 0.05 that 17β-estradiol-treated groups have higher LI than do the corresponding untreated controls.

cells had also increased in size. The increase in cell size (Table 1) is demonstrated by a significant decrease in the mean number of cells per area of culture surface. Similar epithelial cellular morphology, colony shape, and increase in cell size was observed for epithelial cells cultured with GK fibroblasts or CM (Fig. 1b).

When epithelial cells were cultured with control or irradiated fibroblasts, there were several notable differences in their appearance. Firstly as the colonies grew out, their leading edges had distinctive stellate contours (Fig. 1c). Secondly, the epithelial cells did not increase in size (Table 1). Thirdly, after 3 days of culture, advancing strands of epithelial cells were frequently seen growing out from epithelial the colonies (Fig. 2d).

Fibroblast morphology also differed when fibroblasts were grown with epithelial cells. At confluence fibroblasts grown alone ranged in shape from stellate to rounded, and the cells appeared to be randomly oriented (Fig. 2b). This was true for control, irradiated, and GK fibroblasts alike. After 3 days of culture with epithelial cells, control and irradiated fibroblasts appeared more elongated, assumed a more oriented appearance, and were arranged in parallel between the epithelial colonies (Fig. 2c). This change in fibroblast morphology in the presence of epithelial cells was not observed with GK fibroblasts. The different appearance of both epithelial cells and fibroblasts under co-culture conditions again points to the existence of a bidirectional interactive phenomenon between the two cell populations.

DISCUSSION

There appear to be at least two different mechanisms by which mammary fibroblasts can affect estrogen specific responses of normal mouse mammary epithelial cells in vitro. Estrogen-dependent stimulation of PgR does not require the presence of live, metabolically active fibroblasts, since gluteraldehyde-killed fibroblasts were equally effective in promoting the PgR response. Pretreating the culture surface with Type I collagen was also effective, which suggests that fibroblasts may promote the PgR response via a potential extracellular matrix, substratum effect on epithelial cells.

Type I collagen which is produced by stromal cells has been shown previously to influence mammary epithelial cell function in vitro. Emerman et al. (10) first showed that mammary epithelial cells plated on free-floating collagen gels exhibit hormone-dependent morphological and biochemical cytodifferentiation similar to that seen during lactation; these cells, however, do not proliferate. In contrast, cells grown on plastic, while capable of proliferation, do not exhibit similar cytodifferentiation. In organ culture studies of whole mammary gland (which contains both epithelial and stromal cells), the production of type I and III collagen is associated with hormonal induction of lactational differentiation (16). Normal rat mammary epithelial cells grown inside a collagen-gel matrix possess estrogen receptors and exhibit an estrogen-dependent increase in PgR (17). Soluble collagen has also been shown to promote a more normal morphology of corneal epithelium in vitro (18). In this context, it is possible that soluble type I collagen secreted by cultured mammary fibroblasts may be responsible for the promotion of the PgR response that was observed herein with conditioned medium. Epithelial cell shape, production of a basal lamina, and acquisition of polarity are all influenced by collagenous substrata (10). The exact mechanism(s) by which a collagen substratum or other potential extracellular matrix components promote mammary epithelial cell response to estrogen remains to be elucidated.

In contrast to their effect on PgR response, mammary fibroblasts apparently need to be metabolically active and either in close contact with epithelial cells or present in sufficient numbers in order to promote 17β-estradiol-dependent stimulation of epithelial cell DNA synthesis. This is supported by the observations that increased LI is observed upon co-culture with confluent untreated or irradiated fibroblasts or only after 3–5 days of culture if the two cell types are plated together at the same time.
Another important observation is that under the same conditions mammary epithelial cells promote estrogen-dependent increase in fibroblast DNA synthesis. Furthermore, differences in both epithelial and fibroblast cell morphologies are observed when the two cell types are cultured together that are not seen when each cell type is cultured by itself. These results indicate that some form of cooperation between the two cell types occurs; it does not indicate, however, whether only one or both cell types are targets for estrogen action. The means by which this interaction occurs is unknown. Since fibroblast-conditioned medium was not effective in increasing epithelial cell LI, a readily diffusible extracellular factor(s) may not be involved. However, the results do not exclude the possibility that a diffusible factor(s) may only act over short distances or that an effective concentration is reached only when fibroblasts are present in high numbers.

Another possibility is that communication occurs by direct transfer of informational molecules across cell membranes by specialized channels, such as gap junctions (19). These results support the observations of McGrath (8) with regard to a possible requirement for contact between mammary epithelial cells and fibroblasts in order to elicit estrogen-dependent epithelial cell proliferation. Additionally, our results extend the scope of epithelial-fibroblast interactions to include epithelial cell promotion of estrogen-dependent increased DNA synthesis in fibroblasts.

Of particular interest is the observation that the influence of fibroblasts on the two epithelial cell responses to estrogen (PgR stimulation and increased DNA synthesis) can be dissociated. All fibroblast conditions which promote estrogen-dependent increased epithelial DNA synthesis also promote estrogen-dependent PgR increases. However, the reverse is not true. Certain conditions which promote the PgR response such as GK fibroblasts or CM do not promote estrogen-dependent cell increase in LI. The requirement for metabolically active fibroblasts for DNA synthesis but not for the PgR response indicates that the conditions required for DNA synthesis appear to be more stringent than those required for the PgR response.

A dissociation of estrogen-dependent regulation of PgR and estrogen-dependent cell proliferation has been observed in both experimental and human mammary tumors (4, 20, 21). In a number of cases the lack of tumor response does not appear to be explained by a lack of estrogen receptors (20, 21). In vivo the mammary epithelium exists within a complex stromal environment. Mammary tumors can also be heterogenous in composition with respect to epithelial and stromal cells. The results presented here demonstrate that the nature of epithelial-fibroblast interactions can determine and modulate epithelial cell responses to estrogen and may reflect in vivo regulatory processes affecting normal and neoplastic cells.
Fig. 1. Culture morphologies 1 day after plating epithelial cells. a, epithelial cells grown alone; b, epithelial cells grown on confluent monolayers of GK fibroblasts. Note rounded colony shape in both a and b. c, epithelial cells plated on confluent monolayers of untreated, live fibroblasts; epithelial colonies are distinctly stellate in shape, and fibroblasts are seen in contact with epithelial cells but are randomly oriented. d, epithelial cells co-plated with fibroblasts; fibroblasts are randomly oriented, and there is little contact with the epithelial cells. H & E; × 85.
Fig. 2. Culture morphologies after 5 days of culture. a, epithelial cells grown alone at confluence. b, fibroblasts grown alone at confluence; note random orientation of cells. c, epithelial cells grown on confluent monolayer of untreated live fibroblasts; note the parallel orientation of fibroblasts and extensive contact with epithelial cells. d, epithelial cells co-plated with fibroblasts with advancing strand of epithelial cells (arrow). Inset, high magnification of epithelial strand (arrow). a-d, H & E, × 85. Inset, × 200.
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