Cell to Cell Interactions and Normal Mammary Gland Function

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ABSTRACT

The nonepithelial components of the mammary gland are reviewed and their potential for regulatory cell-cell interactions with the epithelial cells are discussed. Studies undertaken to examine the regulatory potential of mammary stromal fibroblasts using an in vitro cell culture system are presented. The influence was examined of epithelial-fibroblast interactions on estrogenic regulation of progesterone receptor concentration in epithelial cells and on epithelial and fibroblast DNA synthesis. Mammary fibroblasts affect estrogen responsiveness in epithelial cells by two different mechanisms. In the case of progesterone receptor regulation, fibroblasts promote estrogen-dependent increases in the receptor via a substratum effect possibly by the production of collagen type I. By contrast, the fibroblast effect promoting estrogen-dependent cell proliferation requires fibroblasts to be metabolically active and in close contact with the epithelium. Additionally, under coculture conditions, estrogen-dependent stimulation of fibroblast DNA synthesis is also observed, indicating a bidirectional, interactive phenomenon between the two types of cells. It is possible that the modulations in epithelial responsiveness to estrogen that are associated with the presence of mammary fibroblasts in vitro reflect regulatory mechanisms that operate in vivo.

INTRODUCTION

The mammary gland is composed of epithelial, adipose, and fibrous connective tissues; the relative proportion of each tissue type varies with the species and developmental state of the gland. Under the influence of a wide range of hormones, the gland undergoes dramatic changes in morphology and function (20). The most readily detectable changes occur in the epithelial compartment of the gland, and the proliferation, morphogenesis, and functional differentiation of the epithelium have been studied almost to the exclusion of all other cell types present. In recent years, there has been a growing interest in more detailed analysis of the cellular composition of the gland, and this has led some to consider that the nature of various cell-cell interactions might provide the basis for important regulatory mechanism(s) in normal mammary gland function. This paper provides a brief review of the various nonepithelial cells types present and what is known about their potential for regulatory interactions with mammary epithelium. The main emphasis of this paper will deal with studies (10, 11) of mammary fibroblast interactions with mammary epithelial cells in culture and their influence on hormonal regulation. Because rodent mammary gland has been studied extensively as a model for delineating hormone action in normal mammary tissue, studies in mice and rats provide the focus for this paper.

In the nonpregnant animal, the epithelial component of the gland is organized into a branching ductal system. The epithelial cells lining the duct are invested with a basal layer of myoepithelial cells and a continuous basement membrane encloses the entire ductal system (Figure 1). In the context of cell-cell interaction, the first nonepithelial cell type to be considered is the myoepithelial cell. The best known function of myoepithelial cells is their facilitation of milk removal during lactation as a result of contractile activity in response to oxytocin (21). However, these cells are directly interposed between the epithelial cells and the basement membrane, i.e., the extracellular...
Figure 1. Representation of the various cells present in normal mammary gland. The epithelial compartment of the gland contains epithelial cells (a) surrounded by myoepithelial cells (b) and separated from the stromal compartment by a basement membrane (c). The stromal compartment consists of the fibroblasts (d) and adipocytes (e); also present are mast cells (f).

Beyond the basement membrane, mammary stromal fibroblasts form a collagenous sheath around the mammary ducts and separate them from the subadjacent adipose tissue (Figure 1). Thus, fibroblasts are the stromal cells in closest proximity to the epithelium. These cells are also responsible for contributing stromal components such as collagen type I to the extracellular space and fibronectin to the basement membrane (27). One can speculate that mammary fibroblasts may play a regulatory role in epithelial function based upon their contribution to the extracellular matrix composition.

The third cell type to be considered is the adipocyte. In the nonpregnant state, the adipose tissue constitutes the major portion of the gland. During pregnancy and lactation, the epithelial component of the gland increases concomitant with a depletion of the adipose tissue. After involution, there is degeneration and loss of the secretory epithelium and the adipose tissue is restored. Thus, cyclic changes in mammary adipose tissue composition occur in relation to mammary gland function. Accumulation of glycogen, the activity of glycogen synthetase, and lipogenic rate in mammary adipocytes are modulated during pregnancy and lactation to channel nutrients to the mammary epithelial cell (2). These changes appear to require the presence of mammary epithelium (2), indicating possible interactive phenomena between the epithelial cells and adipocytes. Recent studies have also shown that linoleate metabolites enhance the in vitro proliferative response of mouse mammary epithelial cells (1). Thus, metabolic cooperation may be a key to adipocyte epithelial cell interactions.

One other cell type that may interact with mammary epithelial cells and play a regulatory role in mammary gland function is the mast cell (Figure 1). Mast cells are ubiquitous in connective tissue; they originate in bone marrow and are similar but not identical to basophils (8). They possess numerous granules that contain a variety of bioactive molecules as well as IgE; these cells are generally associated with the mediation of hypersensitivity reactions. Recent studies on mast cells derived from dimethylbenz[a]anthracene-induced rat mammary tumors indicate that these cells may play a role in hormonal regulation (9). Tumor-derived mast cells can concentrate prolactin in their secretory granules and under appropriate con-

matrix. A recent study (6) has shown that the physical relationship of the myoepithelial cell to the epithelium changes dramatically with different developmental states of the gland. In glands of nonpregnant animals, myoepithelial cells form a complete collar around the ductal epithelium forming linear tracts oriented parallel to the long axis of the ducts, thus effectively separating the epithelium from the basement membrane. By contrast, during pregnancy and lactation, with the development of alveoli, myoepithelial cells assume a stellate appearance, forming loosely woven baskets around individual alveoli, thus leaving large areas of the epithelial cell surface in potential direct contact with the basement membrane. In view of the proposed role of the extracellular matrix in regulation of functional differentiation (3, 15), it is tempting to speculate that myoepithelial cells could have a regulatory role by virtue of their ability to modulate epithelial cell exposure to the extracellular matrix.
ditions prolactin can also be released from the cells. These observations may provide another perspective on mechanisms that could control local concentrations of hormones within mammary tissue.

Studies on embryonic development of the mammary gland have demonstrated that mammary mesenchyme, the stromal precursor, directs and specifies epithelial morphogenesis (22). The adult mammary gland is distinct from many other epithelial organs in that its major morphogenesis occurs postnatally, during pregnancy, and is hormonally regulated (20). Several lines of evidence indicate that the morphogenic processes that occur during pregnancy are similar to those operative during fetal development of the organ and may be dependent upon epithelial-stromal interactions (23, 24).

At present little is known about epithelial-stromal interactions and hormonally regulated growth and function in the adult mammary gland. This question is difficult to approach in vivo in the whole animal, and thus, this problem was approached in vitro using a cell culture system. In vivo, estrogen is an important hormone for regulation of growth and function of mammary gland; it promotes mammary epithelial cell proliferation and regulates mammary progesterone receptor (PgR) concentration (12, 13, 20). Hormone receptors for estrogen (ER) are localized in the mammary gland and are present in both the epithelial and stromal compartments of the gland (14). Of further interest, ER concentration varies in stromal and epithelial tissues depending on the developmental state of the gland (Table 1). For these reasons, attention was focused on estrogen action in relation to mammary epithelial-stromal cell interactions. Initial studies (10, 11) addressed specifically the interaction of the mammary fibroblast with the epithelium and examined the response of these cells to estrogenic regulation of cell proliferation and PgR concentration.

**MATERIALS AND METHODS**

**Cell Culture**

Balb/C mice 2 to 5 mo of age and 14 to 18 d pregnant were the source of mammary tissue. The tissue was dissociated according to previously described methods to obtain mixed cultures containing epithelial cells and fibroblasts (11). The two cell types were separated (10) to produce either epithelial cultures or fibroblast cultures. The epithelial cell and fibroblast cultures were determined to be 97 and 100% pure, respectively, using immunocytochemical methods (11); antibody directed against vimentin was used to identify fibroblasts (17). All cell types were cultured in Medium 199 containing 5% charcoal-stripped fetal calf serum (CS-FCS), 0.006 μg/ml insulin, 1 μg/ml prolactin, and 10⁻⁸ M cortisol. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂ in air; medium was changed every 2 d. To determine the effect estrogen on PgR concentrations or DNA synthesis cells were cultured with or without 20 nM 17β-estradiol.

Fibroblasts cultures that were used for coculture experiments were plated 1 wk prior to

<table>
<thead>
<tr>
<th>Developmental state¹</th>
<th>Intact mammary gland specific [³H]E₂ binding</th>
<th>Epithelium-devoid stroma specific [³H]E₂ binding</th>
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<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
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<tr>
<td>Virgin (2 to 5 mo)</td>
<td>1733</td>
<td>277</td>
</tr>
<tr>
<td>Pregnancy (14 d)</td>
<td>1023</td>
<td>153</td>
</tr>
<tr>
<td>Lactation (7 to 10 d)</td>
<td>787</td>
<td>118</td>
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¹In all cases mice were ovariectomized, or ovariectomized plus hysterectomized (pregnant mice) 5 to 7 d prior to assay to remove endogenous estrogens. Thus measured = total cytoplasmic estrogen receptor. Estrogen receptor concentration was determined by Scatchard analysis. For methods see (14). E₂ = Estradiol.
the addition of the epithelial cells. The fibroblasts received treatments to render them either nonproliferative by irradiation or nonviable by gluteraldehyde fixation as previously described (10). To obtain conditioned medium, fibroblast cultures were incubated for 24 h in Medium 199 without FCS or hormones and processed and stored as described (10).

Steroid Hormone Receptor Assay

Intact cells grown for indicated lengths of time were assayed while still attached to culture dishes. To measure ER or PgR the cells were incubated with either $[^3H]$estradiol (1 to 10 nM) or $[^3H]$R5020 (1 to 12 nM) with or without 100-fold excess radioinert estradiol or R5020 and processed as previously described (10). All binding data were analyzed by the method of Scatchard (25). The DNA content was determined according to the method of Ceriotti (4).

Measurement of Deoxyribonucleic Acid Synthesis

Cells were plated, in parallel, on glass coverslips, and autoradiography was as described (19) using $[^3H]$thymidine (6.7 Ci/mmol). Labeling was for 1 h. Labeled nuclei were counted in 20 random high power (400x) microscope fields with a minimum of 1000 of each cell type counted (10).

Statistical Analysis

All data are expressed as the means ± SE and were analyzed for significance using Student's t-test or analysis of variance where appropriate.

RESULTS

Analysis of Estrogen Responsiveness in Mixed Versus Epithelial Cell Cultures

Initial experiments were carried out with mixed cultures containing both epithelial cells and fibroblasts in order to determine if this model system would exhibit an in vitro response to estrogen. The effects of estrogen treatment on PgR concentration are shown in Figures 2 and 3. Soon after plating the cells (Figure 2), when cultures were still subconfluent, estrogen was only moderately effective in increasing PgR concentration. However, when confluent cultures were treated with estrogen (20 nM) there was almost a threefold increase in PgR concentration after 3 to 5 d of treatment. This result is very comparable to the two- to threefold stimulation of PgR observed after in vivo administration of estradiol to ovariectomized virgin (12) or pregnant mice (13). The observed effect of estrogen on PgR concentration appears to specific for estrogenic compounds (Figure 4) since estradiol and diethylstilbestrol were effective in increasing PgR concentration and the antiestrogen, tamoxifen ($10^{-6}M$), was able to block the estrogenic effect; further evidence of specificity is indicated by the lack of effect obtained with 20 nM progesterone or dexamethasone.

Having established that mixed cultures exhibit a characteristic PgR response to estrogen,
Figure 3. Time course of estrogen stimulation of progesterone receptor concentration in confluent cultures of mixed cells. Cells were grown to confluence in the absence of estradiol, changed to medium with (o) or without (●) 20 nM estradiol, and assayed for progesterone receptors (PgR) 1, 3, or 5 d later (11). *P = .001 in estrogen-treated groups; PgR are higher than in untreated controls.

Figure 4. Steroid hormone specificity of progesterone receptor stimulation. Confluent mixed cells cultured without estradiol were changed to medium without estradiol (C), or 20 nM estradiol (E₂), or diethylstilbestrol (DES), or dexamethasone (DEX), or progesterone (Pg), or 10⁻⁶ M tamoxifen (T), or estradiol plus tamoxifen (E₂ + T).

Figure 4. Steroid hormone specificity of progesterone receptor stimulation. Confluent mixed cells cultured without estradiol were changed to medium without estradiol (C), or 20 nM estradiol (E₂), or diethylstilbestrol (DES), or dexamethasone (DEX), or progesterone (Pg), or 10⁻⁶ M tamoxifen (T), or estradiol plus tamoxifen (E₂ + T).

it was of interest to determine if purely epithelial cultures responded similarly. No stimulation of PgR was observed in the epithelial cultures (Figure 5). One possible explanation for the lack of response in epithelial cultures was that the increase in PgR concentration was occurring in the fibroblast population of mixed cultures. To investigate this possibility, fibroblast cultures were examined for the presence of PgR and for the ability of estrogen to increase PgR concentration. In all cases, Scatchard analysis of [³H]R5020 binding revealed only low affinity binding that did not satisfy the criteria for receptor binding (Figure 6). Furthermore, treatment with estrogen had no effect on the quality or quantity of the binding.

Estrogenic regulation of PgR concentration in mammary tissue is thought to be regulated via an ER-mediated mechanism (16). Thus, the lack of estrogen responsiveness observed in the epithelial cultures was possibly due to a loss of ER. To test this possibility, epithelial, fibroblast, and mixed cultures were assayed for ER at various times after plating. Concentrations of ER in the three types of cultures were similar, and in all cases, ER concentrations were maintained equally well over time (Table 2). From these studies we conclude that one specific mammary epithelial cell response to estrogen in vitro, namely PgR regulation, is associated with the presence of mammary stromal fibroblasts.

Analysis of the Fibroblasts Influence on Epithelial Cell Responsiveness to Estrogen

The favorable effect of fibroblasts on mammary epithelial cells could be explained in several different ways. One possibility is that fibroblasts provide some undefined soluble factor that is required for epithelial response to estrogen. In this context, conditioned media from cultured mouse fibroblasts have been reported to stimulate normal mouse mammary
Figure 5. Comparative effect of estrogen treatment on progesterone receptor concentration in mixed versus epithelial cultures. Confluent epithelial (□) or mixed (●) cultures were changed to medium with (E₂) or without (C) 20 nM estradiol and assayed for progesterone receptors (PgR) 3 d later (11). *P<.01 that PgR concentration in estrogen-treated group is greater than in controls.

Table 2. Concentration of specific [3H]-estradiol (E₁) binding sites in epithelial, fibroblast, or mixed cultures.

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<thead>
<tr>
<th>Day</th>
<th>Epithelial</th>
<th>Mixed</th>
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<tr>
<td></td>
<td>K_d (x 10^9 M)</td>
<td>K_d (x 10^9 M)</td>
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<td></td>
<td>(fmol/mg DNA)</td>
<td>(fmol/mg DNA)</td>
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<tr>
<td>3</td>
<td>1.94±0.52</td>
<td>1.88±0.49</td>
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<tr>
<td>5</td>
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<tr>
<td>7</td>
<td>1.46±0.25</td>
<td>1.33±0.21</td>
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Figure 6. Scratchard analysis of [3H]-R5020 binding in fibroblast cultures. Confluent cells were changed to medium with (□) or without (●) 20 nM estradiol and assayed 3 d later (13). B/F = Bound-to-free ratio.

epithelial cell proliferation in vitro (7). However, the physical nature and chemical composition of the culture substratum are also known to be important for mammary epithelial cell function in vitro (5). Thus, fibroblasts might function to improve the culture substratum. Alternatively, direct contact between epithelial cells and fibroblasts may be required to facilitate estrogen responsiveness (19). The purpose of the following series of experiments was to distinguish among these described possibilities and to elucidate the mechanism(s) by which fibroblasts might influence epithelial cell responsiveness. Observations include fibroblast effects on estrogen dependent epithelial cell DNA synthesis.

**Progesterone Receptor Regulation**

To investigate the nature of fibroblast influence on the PgR response a number of fibroblast conditions were created and they were cocultured with epithelial cells 7 d later. The results are shown in Figure 7. Fibroblasts were either control untreated, lethally irradiated, gluteraldehyde-killed (GK) or epithelial cells were cultured in the presence of conditioned medium (CM) from fibroblasts. All of the conditions tested were about equally effective in promoting estrogen-dependent increases in PgR concentration. From these results one could conclude that fibroblasts need not be alive or even physically present since conditioned medium was also effective. Fibroblasts may be influencing the epithelial cells via a substratum effect. Pretreating culture dishes with collagen type I, an extracellular product of fibroblasts, was also effective in promoting the PgR response providing further evidence for a substratum effect (Figure 7).

**Regulation of Deoxyribonucleic Acid Synthesis**

To determine the nature of fibroblast influence on epithelial cell proliferation, DNA synthesis was measured in parallel cocultures, autoradiographically. The results of these studies are shown in Figure 8. When epithelial cells were grown alone, estrogen had no stimulatory effect. In contrast, coculture with live fibroblasts in the presence of estrogen resulted in a significant increase in the labeling index (LI, percent labeled nuclei). Coculture with irradiated fibroblasts also resulted in a significant increase in LI on d 1; however, after 5 d of culture a stimulatory effect was not observed. Coculture with GK fibroblasts was not effective. Conditioned medium also failed to promote epithelial cell DNA synthesis. It was possible that the presence of estrogen in fibroblast cultures may have been necessary to produce a stimulatory soluble factor, so CM was also obtained from fibroblasts cultured with estrogen; no stimulatory effect was observed with this CM either.

Because coculture with either untreated or irradiated fibroblasts were the only conditions that facilitated estrogen-dependent epithelial cell DNA synthesis, this suggested that in addition to being metabolically active, that the fibroblasts needed to be in close contact with the epithelial cells. To determine if this was
Figure 8. Effect of fibroblast treatment on estrogen-dependent stimulation of epithelial cell DNA synthesis. Epithelial cells were either cultured alone (E) or with mammary fibroblasts that were untreated (F1), irradiated (IF), or gluteraldehyde-killed (GK) or with conditioned medium for fibroblasts cultured with (E + CM) or without (CM) 20 nM estradiol; epithelial cells were also cocultured with fibroblasts (F2). In all cases, epithelial cells were plated with or without (●) 20 nM estradiol and labelling indices were determined 1 (a) or 5 (b) d later (10). **P<.05 that cocultures treated with estrogen have higher LI than do corresponding control cultures.

SUMMARY AND CONCLUSIONS

Using a cell culture model system, mammary gland cell-cell interactions and their potential regulatory role in normal gland function have been investigated, specifically, the nature of mammary epithelial cell interactions with mammary stromal fibroblasts in relation to estrogen-regulated DNA synthesis and PgR concentration.

There appear to be at least two different mechanisms by which mammary fibroblasts affect estrogen specific responses in the epithelial cell. In the first case, estrogen-dependent stimulation of PgR appears to be mediated by fibroblasts via a potential extracellular matrix, substratum effect. Type I collagen, which is produced by fibroblasts, is one potential
candidate for the molecular mediator of the observed effect. In recent years, there have been numerous reports that expression of differentiated function of mammary epithelial cells can be critically influenced by the culture substratum and as herein, collagen type I has been implicated (26). Epithelial cell shape, production of a basement membrane, and acquisition of polarity are all influenced by collagenous substrata (5). The exact mechanism(s) by which collagen or other extracellular matrix components regulate mammary epithelial response to mammagenic hormones such as estrogen remains to be elucidated.

In the second case, mammary fibroblasts need to be metabolically active and either in

Figure 9. Culture morphologies 1 d after plating epithelial cells. a) Epithelial cells alone; b) epithelial cells on confluent monolayers of gluteraldehyde-killed fibroblasts. Note rounded colony contours in both a and b. c) Epithelial cells plated on confluent monolayers of untreated, live fibroblasts; epithelial colonies display stellate contours and fibroblasts are seen in contact with epithelial cells but are randomly oriented. d) Epithelial cells coplated with fibroblasts; fibroblasts are randomly oriented and there is little contact with the epithelial cells. H&E, ×85.
close contact with epithelial cells or present in sufficient numbers in order to promote estrogen-dependent DNA synthesis. Additionally, under the same conditions, mammary epithelial cells promote estrogen-dependent DNA synthesis in mammary fibroblasts. Furthermore, differences in morphologies of the two cell types are observed under coculture conditions that are not seen when each cell type is cultured by itself. These results strongly indicate some form of cooperation between the two cell types. The mechanism(s) involved in this interaction also remain to be elucidated. Despite the finding that fibroblast-conditioned medium was ineffective in this case, the possibility still remains that a soluble factor(s) is produced that

Figure 10. Culture morphologies after 5 d 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, untreated fibroblasts; note parallel orientation of fibroblasts and extensive contact with the epithelial cells. d) Epithelial cells coplated with fibroblasts with advancing strand of epithelial cells (arrow). Inset, high magnification of epithelial strand (arrow), a to d, H&E, X 85. Inset X 200.
is effective only 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 (18).

In vivo the mammary epithelium exists within a complex stromal environment. Along with the mammary stromal fibroblast are a number of other cell types that may have potential regulatory roles in normal mammary gland function. Advancement of our knowledge in the area of cell-cell interactions in the mammary gland may provide important insights into mechanisms that regulate cell proliferation as well as the synthesis and secretion of milk constituents during lactation.

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