Ovarian steroids and the human breast: Regulation of stem cells and cell proliferation

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Abstract

Ovarian steroidal control of mammary gland proliferation and differentiation is not well defined in the human. We therefore developed the athymic nude mouse model in which intact normal human breast tissue is xenografted subcutaneously and treated with human physiological serum levels of oestrogen (E) and/or progesterone (P). We showed that: (i) E, and not P, is the major steroid hormone inducing proliferation of epithelial cells in the adult non-pregnant, non-lactating breast; (ii) E induces progesterone receptor (PR) expression; and (iii) PR expression is maximally induced at low E concentrations while a higher amount of E was required to induce proliferation. Using double label immuno-fluorescence, we demonstrated that cells expressing the oestrogen receptor-α (ERα) invariably contained the PR but that steroid receptor expression and cell proliferation (Ki67 antigen) were dissociated. Recently, we have demonstrated that some ERα/PR-positive epithelial cells are quiescent breast stem cells suggesting that they act as “steroid hormone sensors” that secrete paracrine factors to regulate the proliferative activity of adjacent ERα/PR-negative epithelial cells. The dissociation between steroid receptor expression and cell proliferation in normal epithelium was lost at an early stage in ERα/PR-positive breast tumour formation perhaps indicating that they arise from deregulation of the normally quiescent breast stem cells.

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Keywords: Breast; Steroid receptors; Proliferation; Paracrine signal; Stem cells

1. Introduction

Ovarian steroids are absolutely necessary for the development, proliferation and differentiation of the normal human mammary gland [1]. There is epidemiological evidence that ovarian hormones alter the risk of breast cancer. For example, an early menarche and a late menopause increase breast cancer risk, while an early menopause protects against breast cancer. This suggests that the risk of breast cancer relates to cumulative exposure to ovarian hormones [2–4]. The greater risk from an increased reproductive lifespan may relate to the total number of times that the breast epithelium undergoes cyclical proliferation in response to ovarian hormones, which increases the chances of cancer initiation and promotion [5,6].

It is clear from epidemiological studies that both pregnancy and breast-feeding are protective in terms of breast cancer risk [7]. This protective effect was thought to be related to the full lactational differentiation that the breast epithelium undergoes in response
to hormones during pregnancy [8], but recent studies in rodents suggest that it is exposure to pregnancy hormones rather than differentiation that is the protective factor [9]. However, we still do not completely understand how hormones regulate normal human breast development, proliferation and differentiation, and how their effects on normal human breast epithelial cells relate to breast cancer risk. The purpose of this review is to describe the hierarchical cellular organisation of human breast epithelial tissue and its regulation by ovarian steroids.

2. Breast development

The breast is an unusual organ in that much of its development occurs during puberty or in the adult during pregnancy and lactation. The adult female breast consists of a branching, tree-like network of ducts lined by a double layer of epithelial cells that is surrounded by delimiting fibroblasts and embedded in an extracellular matrix [10]. The rudiments of the gland are developed during embryogenesis when newly formed breast epithelial cells become indented at the epithelial-stromal border, sprouting and separate into 10–15 branches of the epithelial ducts that open separately onto the epidermal surface at the nipple. At puberty, the network of ducts leading from the nipple grows and divides into bundles of primary and secondary ducts lined with epithelial cells and ending with end bud structures [11]. It is from the end buds and ductal side branches that the terminal duct lobulo-alveolar units (TDLU), or lobules, form. These are the principal, functional milk-producing units of the breast. These lobules exist initially as alveolar buds that mature following menarche into a variable number of blind-ending, secretory sacs known as acini, alveoli or ductules which open into the intra-lobular terminal duct. The TDLU is the site from which many epithelial hyperplasias and carcinomas of the breast are thought to arise since this is where they are most often observed histo-pathologically [12]. In the mouse, oestrogen induces growth of the ductal system during puberty while progesterone stimulates growth of the lobules during pregnancy [13–16]. In contrast, human breast lobules form during and following puberty, and it is therefore not immediately clear which ovarian steroid regulates development of the human TDLU [1].

Breast development achieves full maturity and function during pregnancy and lactation. The full development of the TDLUs is accelerated during pregnancy as the breast lobules expand in terms of the number of epithelial cells and alveoli that they contain in preparation for lactation. At the termination of lactation, the lobules involute to resemble those present in the non-pregnant gland, although they may retain a larger number of individual alveoli per lobule than before [10,11].

3. Epithelial cell phenotypes

The terminal ducts and tubular alveoli within the lobules are lined by an inner layer of luminal epithelial cells surrounded by an outer layer of basal or myoepithelial cells and the basement membrane that separates them from the intra-lobular stroma. In the non-pregnant gland, the myo- and luminal epithelial cells are distinguished not only by their relative positions, but by the proteins that they express. The myo-epithelium expresses a distinct subset of epithelial cytokeratins (CK 5 and 14), the common acute lymphoblastic leukaemia antigen (CALLA) and smooth muscle actin [17–19]. In contrast, the luminal cell type can be distinguished by expression of a subset of epithelial cytokeratins (CK 8, 18 and 19), nuclear receptors for the ovarian steroid hormones oestrogen and progesterone and low (but detectable) levels of milk proteins [17,18,20,21]. The luminal cells also account for more than 90% of epithelial cell proliferation that is observed in the non-pregnant gland [22,23]. Significantly, more than 90% of breast tumours express cytokeratins distinctive of the luminal phenotype, and greater than 75% express steroid receptors, indicating that the luminal cell type is the major target for breast tumourigenesis [24,25].

4. Ovarian steroid hormones and regulation of epithelial proliferation

In the adult, non-pregnant, non-lactating breast, epithelial proliferation is maximal approximately one week after ovulation during the luteal phase of the menstrual cycle. This is when both estrogen (E) and progesterone (P) are being secreted by the corpus luteum
The data on breast contrast with those from the endometrium where oestrogen drives proliferation during the follicular phase and have led to the conclusion that, in the breast, P is the major breast mitogen, possibly after E priming. To study this assumption, we developed a model in which small pieces of intact normal human breast tissue were implanted subcutaneously into adult female athymic nude mice. Intact pieces of tissue were used in order to preserve the architecture of the tissue so that the epithelium and stroma remained in contact. Two weeks after tissue implantation, stilastic pellets containing steroid hormone were inserted subcutaneously at the base of the tail. These pellets were tailored to give serum concentrations equivalent to those seen in the follicular or luteal phases of the menstrual cycle [34].

The human breast xenografts were removed 1, 2 and 3 weeks after the start of treatment and the percentage of proliferating epithelial cells was determined either by tritiated thymidine incorporation during S-phase followed by autoradiography, or by immunohistochemical staining using the Ki67 antibody which recognises a proliferation-associated nuclear antigen. In tissue removed from untreated control mice or those treated with luteal phase levels of progesterone, breast epithelial proliferation rates were very low (Fig. 1). Follicular phase E levels induced low levels of proliferation while luteal phase levels of E significantly increased proliferation. The addition of P to luteal phase E levels had no additional effect in this model. E alone therefore appeared to explain the effect of the menstrual cycle on breast proliferation since no effects of P were observed. We therefore investigated whether there were more subtle effects of P on proliferation by combining different concentrations of the two hormones. No effect of luteal P levels on low, follicular E levels was observed. The conclusion from this study was that a low dose of E equivalent to follicular phase levels induced some proliferation, but higher dose luteal phase levels were necessary to maximally induce cell division and there were no obvious effects of P [34–36].

Our experimental studies in the athymic nude mouse model indicated that oestrogen is a prime inducer of breast epithelial cell proliferation and may regulate the cyclical variation in breast cell proliferation during the menstrual cycle. E has also been reported to induce ductal elongation during puberty in the mouse and expression of the receptors for P, a prerequisite for its activity [13,14]. P is certainly involved in the ductal side-branching and alveolar development that occurs during pregnancy in the mouse mammary gland [15,16]. However, in the human, alveolar development occurs during puberty and therefore no strict parallel can be drawn [10,11].

The data presented above on the effects of P in human breast epithelium implanted into the athymic nude mouse model are not in agreement with several recent publications on the risk of breast cancer in relation to hormone replacement therapy (HRT). These indicate that it is the combination of E and P in long term HRT that correlates most strongly with an increased risk of breast cancer [37–40]. Two groups have investigated this further by looking at the proliferation rates of normal breast epithelium removed from post-menopausal women on E only, or E + P HRT and comparing them to untreated post-menopausal women of a similar age [41,42]. A clear effect of the length of HRT administration was observed. Less than 5 years use of HRT of any type had no effect on proliferation. In contrast, more than 5 years on combined E + P HRT significantly increased breast epithelial prolifera-

![Fig. 1. The graph demonstrates the effect of human physiological serum levels of E on the epithelial proliferation of normal breast tissue xenografted into the athymic nude mouse. Proliferation was measured by incorporation of 3H-dT and autoradiography. The medians (bars) and interquartile ranges (columns) of the percentage-labelled cells are shown for treatments (n = 10 per treatment group). (a) C = control (untreated); P = 7 days progesterone (4 mg pellet) treatment; E = 7 days oestradiol (Lo-0.5 mg pellet, Hi=2 mg pellet) treatment; (+) significantly different from C (P<0.01). (*) Significantly different from 2 weeks untreated control (P<0.01).](image-url)
The increased proliferation seen in normal breast epithelium taken from post-menopausal women being treated with combination E + P HRT correlates to increased breast cancer risk. Since the risk of breast cancer may be linked to the proliferation of normal epithelium, these data suggest that long term P treatment has an effect in the breast that we have not observed in our model of short-term treatment. We conclude from this that either P has different effects in post-menopausal breast, especially when given as a long-term treatment.

Alternatively, the form of P may be important. For example, it has been shown that 19-norprogestins that are used in some HRT formulations can induce proliferation in breast cell lines by directly activating the ERs [43–45]. Secondly, it has been reported that the endogenous enzyme 5α-reductase can convert P to a 5α-metabolite that stimulates the growth of breast cancer cells in culture [46].

5. Steroid receptors: cellular sensors of hormonal cues

Classically, E and P exert their effects by binding to receptors in the nucleus of the cell and altering the receptor structure such that they efficiently bind specific DNA sequences within gene promoter regions termed steroid response elements. The ER and the PR are thus essentially ligand-activated transcription factors that regulate gene expression in the presence of the steroids [47]. The cells that express the classical ER and the PR are found within the luminal epithelial, but not the myoepithelial or stromal, cells of the human breast [20]. In the last decade, a second ER-like gene has been discovered; the classical ER has been renamed the ERα, the novel form is ER-β [48–50]. The ERαs and the PR are known to be co-localised in approximately 20% of luminal epithelial cells [51]. The ERβ appears to be much more widely expressed than ERαs in normal breast tissue, being detected in the majority of luminal epithelial and myoepithelial cells together with stromal fibroblasts and endothelial cells [52–54]. The PR is also expressed as two different protein isoforms PRA and PRB, although both of these are transcribed from the same gene using different promoters. PRB is the longer version containing an additional 164 amino acids at the N-terminal of the protein [55]. In vitro data support the view that PRB is the active PR, while PRA is either inactive or acts as an inhibitor of PRB [55]. However, in the normal physiology of breast epithelium, both PRA and PRB appear to be co-expressed in the same sub-set of cells and at similar levels [56].

In normal human adult breast tissue, luminal cells co-expressing both ERαs and PR, are distributed evenly throughout the intra-lobular ducts and peripheral alveoli [20,33,51,57]. ERα expression levels in normal epithelium are inversely correlated with age such that the proportion of ERα-positive cells is lower in pre-menopausal compared to post-menopausal tissue [58]. In the pre-menopausal breast, approximately 5% of epithelial cells are proliferating in response to steroid hormones but, surprisingly, these cells do not contain ERαs but are often adjacent or in close proximity to those that do [51]. This dissociation between steroid receptor expression and cell proliferation in the mammary epithelium has since been demonstrated by other groups [59–62]. Since it has been shown that receptors are not simply being down-regulated during cell division, these data suggest a model where ovarian steroids stimulate proliferation via paracrine signals secreted by ERαs-positive cells (Fig. 2). For example, mouse mammary epithelium in which the ER gene has been deleted (ERKO mice) fails to undergo alveolar development. However, this growth deficit in response to E can be overcome by mixing ER KO cells together with wild type cells before transplantation into the cleared fat pad of syngeneic hosts [63]. This suggests that the wild type cells communicate with the ER KO cells via paracrine growth signals. We have recently carried out microarray analyses to identify the growth signals induced by E in human breast implanted into our athymic nude mouse model. Previously identified E-induced genes such as PR and pS2 were amongst the most highly up-regulated, along with genes encoding the growth factor amphiregulin, intracellular pathway molecules (that mediate signalling from cell surface receptors) such as MAP kinase, JAK and STAT, transcription factors that induce growth such as Myc, Myb and ETS and the cell cycle gene cyclin D1 (Fig. 2) [64]. Amphiregulin, which binds the epidermal growth factor receptor, provides a good candidate for the molecule that relays the paracrine signal generated in the human breast in response to E from the ERα-positive cell to an adjacent cell that proliferates. More recently, we reported...
that the separation between steroid receptor expression and cell proliferation observed in the normal mammary epithelium is altered at an early stage in human breast tumourigenesis such that E directly drives cell proliferation in ER-positive cancers [65]. This alteration in E action may increase the sensitivity of breast tumour cells to E and explain why E-dependent breast tumours arise post-menopausally at a time when the normal epithelium undergoes a process of involution as ovarian steroid levels decline.

6. Steroid receptor-positive cells include a population with stem cell characteristics

Breast epithelial stem cells are thought to be the primary targets in the aetiology of breast cancer. Since breast cancers mostly express oestrogen and progesterone receptors (ERs and PR), we examined the biology of these ERs/PR-positive cells and their relationship to stem cells in normal human breast epithelium. We employed several complementary approaches to identify putative stem cell markers, to characterise an isolated stem cell population and to relate these to cells expressing the steroid receptors ERs and PR. Using DNA radiolabelling in human tissue implanted into athymic nude mice, a population of label-retaining cells were shown to be enriched for the putative stem cell markers p21^{(CIP1)} and Msi-1, the human homolog of Drosophila Musashi. Steroid receptor positive cells were found to co-express these stem cell markers together with cytokeratin 19, another putative stem cell marker in the breast [66]. Human breast epithelial cells with Hoechst dye-effluxing "side population" (SP) properties characteristic of mammary stem cells in mice were demonstrated to be undifferentiated "intermediate" cells by lack of expression of myoepithelial (CALLA) and luminal (MUC1) epithelial membrane markers. These SP cells were six fold enriched for ERs-positive cells and expressed several fold higher levels of the ER, p21^{(CIP1)} and Msi1 genes than non-SP cells. In contrast to non-SP cells, SP cells formed branching structures in matrigel which included cells of both luminal and myoepithelial lineages. The data suggest a model where scattered steroid receptor-positive cells are stem cells that self-renew through asymmetric cell division and generate patches of transit amplifying and differentiated cells [67]. Many putative stem cell

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<th>Putative stem cell markers in the normal mammary gland</th>
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<td>Sca-1</td>
<td>Mouse</td>
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<td>p21</td>
<td>Human</td>
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<td>Musashi</td>
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<td>α6 integrin (CD49f)</td>
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markers have been reported for the normal mammary gland and these have summarised in Table 1.

7. Summary

Over the years, there has been controversy about which of the ovarian steroids, E and P, regulates breast epithelial cell proliferation and consequently, breast cancer risk. Our data and those of others lead us to the conclusion that E is the major mitogen in the non-pregnant, pre-menopausal breast, whereas P may have a more significant long term role in the post-menopausal breast where E levels are much lower. Clearly, recent reports on E + P HRT indicate that P contributes to breast tumourigenesis. Breast epithelium does not appear as sensitive an E target organ as the endometrium, and our data suggest that this decreased steroid responsiveness may be due to an indirect effect on proliferation which requires paracrine factors to mediate their signal. Recent evidence for this and the preliminary characterization of stem cells in breast epithelium suggest that an increased understanding of normal breast biology may provide us with new opportunities for the prevention of breast cancer through specific targeting of the cell population that is susceptible to cancer.

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