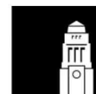

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ORIGINAL ARTICLE

Distinct expression patterns of ERα and ERβ in normal human mammary gland

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Aim: Two oestrogen receptors (ERs) have been identified to date—the "classic" ERα and the more recently described ERβ. Although much is known about ERα at the mRNA and protein levels, our knowledge of the expression and distribution of ERβ protein is much more limited. The aim of this study was to compare the cellular distribution of ERα and ERβ in normal human mammary gland.

Methods: Formalin fixed, paraffin wax embedded material was obtained from reduction mammoplasty specimens, normal tissue adjacent to breast tumour, or fibroadenoma. Sections were immunohistochemically stained for ERα, ERβ, and the progesterone receptor. The staining pattern for each antibody was evaluated and compared.

Results: ERα was restricted to the cell nuclei of epithelial cells lining ducts and lobules. Although ERβ was also seen in these cells, additional strong staining was detected specifically in the cell nuclei of myoepithelial cells. Occasional staining was seen in surrounding stromal and endothelial cell nuclei and in lymphocytes.

Conclusions: ER subtypes have distinct distribution patterns in the normal mammary gland. The widespread distribution of ERβ suggests that it may be the dominant ER in the mammary gland where it may be acting as a natural suppressor.

Towards the end of the last century, a second oestrogen receptor (ER) was identified. To distinguish it from the original receptor, now re-christened ERα, the new receptor was called ERβ.[1 2] ERs are ligand activated transcription factors, which mediate the effect of oestrogens in steroid target tissues. The genes encoding the two types of receptor are located on different chromosomes: ERβ has been mapped to chromosome 14q22–24,[3] whereas ERα is located on chromosome 6q25.1.[4] Although they are the product of independent genes, they share homology at the DNA and ligand binding domains (96% and 58%, respectively[2]). Both receptor subtypes bind oestrogens with a similar affinity and activate the expression of reporter genes containing oestrogen response elements in an oestrogen dependent manner.[5]

Oestrogens are necessary for the development and maturation of the mammary gland. However, immunohistochemical analysis of breast tissue from premenopausal women has estimated that less than 20% of luminal epithelial cells express Erα.[6–8] Curiously, double immunolabelling experiments in both human and murine mammary gland have shown that cells that are immunopositive for ERα very rarely undergo proliferation, as determined by the lack of expression of the cell cycle associated antigens Ki-67 and proliferative cell nuclear antigen, or failure to incorporate ^{3}H-thymidine.[7–9] It has been hypothesised that the lack of proliferation seen in ERα positive cells may indicate a hierarchical organisation, whereby the proliferation of ERα negative cells is under the control of paracrine factors released from their ERα positive counterparts.[8] However, the discovery of ERβ opens up the possibility that cells originally considered ERα negative may in fact be expressing ERβ. The presence of ERβ has been demonstrated by reverse transcription polymerase reaction (RT-PCR) in the normal mammary gland, where it was frequently detected.[10 11] This was in contrast to breast tumours where the expression of ERβ is usually seen only in combination with ERα.[10 12] However, RT-PCR is limited because it cannot provide information on cellular distribution patterns. Therefore, the aim of our study was to evaluate the pattern of expression and distribution of both ER subtypes in archival, paraffin wax embedded, normal human mammary gland and to correlate this with the expression of the ER regulated progesterone receptor (PR).

> "Both receptor subtypes bind oestrogens with a similar affinity and activate the expression of reporter genes containing oestrogen response elements in an oestrogen dependent manner"

METHODS

With approval from the local ethics committee, archival paraffin wax embedded material from normal breast adjacent to tumours (n = 65), reduction mammoplasty material (n = 30), or fibroadenoma (n = 2) was obtained. Serial sections (4–5 μm thick) were mounted on to superfrost plus slides (BDH, Poole, Dorset, UK), dewaxed in xylene, and rehydrated through graded alcohols. To unmask antigenic sites, slides were immersed in 1mM citrate buffer, pH 6, and microwaved at full power (600 W) for 27 minutes for ERα and ERβ, and 10 minutes for PR. After cooling, sections were then incubated with the relevant monoclonal antibodies. For ERα, the monoclonal antibody 1D5 (Dako, High Wycombe, UK) was applied at a dilution of 1/50 for one hour at room temperature. For ERβ, the monoclonal antibody 14C8 (Abcam, Cambridge, UK) was applied at a dilution of 5 μg/ml overnight at 4°C. Validation of this antibody has previously been published by our group.[12] The antibody was affinity purified and raised by immunising mice with a recombinant protein encoding 1–153 amino acids of the human ERβ sequence. According to the manufacturers, there is no crossreactivity of 14C8 with human ERα or ID5 with human ERβ. For PR, the monoclonal antibody PgR636 (Dako) was used at a 1/50 dilution for one hour at room temperature. After incubation with the

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; RT-PCR, reverse transcription polymerase reaction

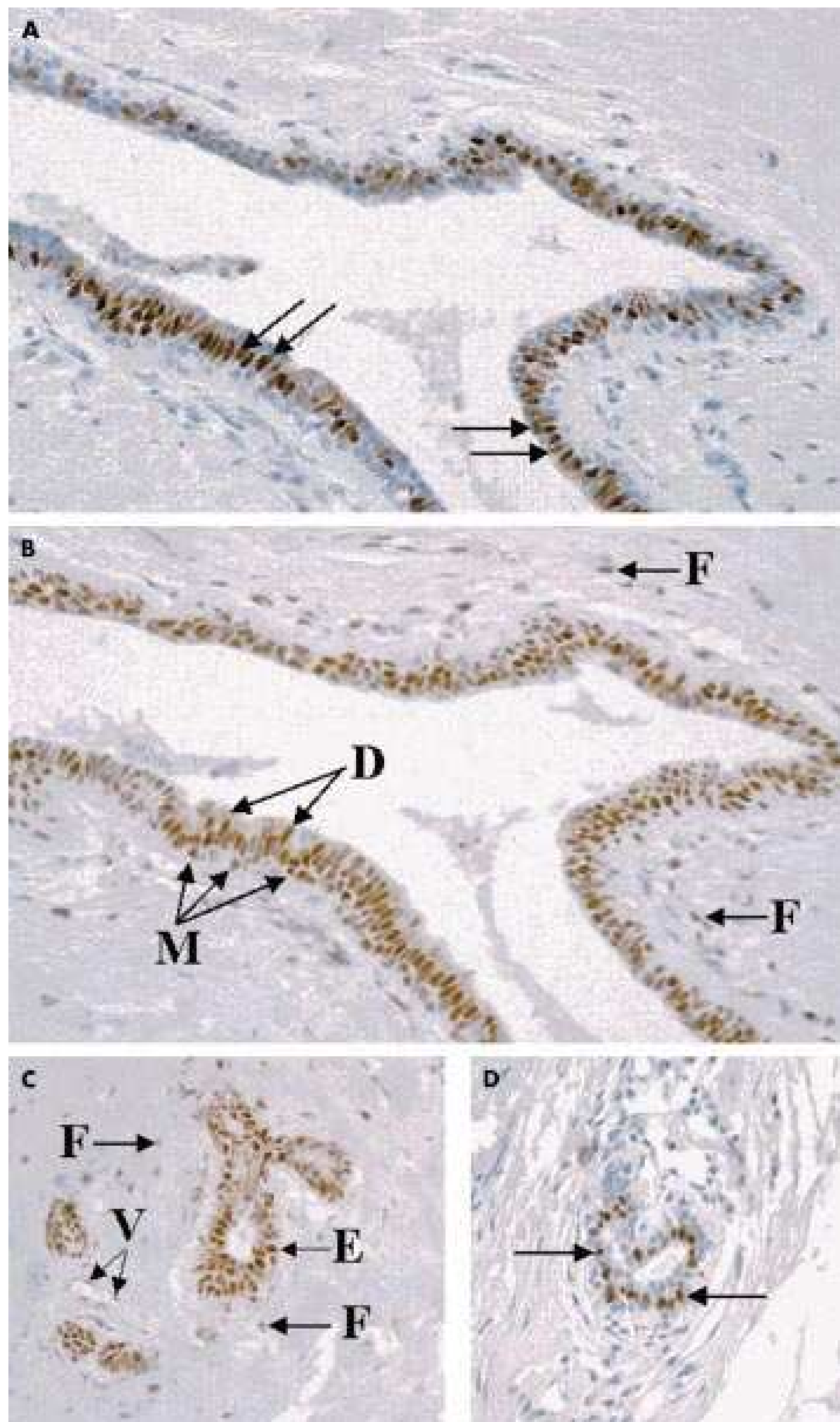

Figure 1 (A) Expression of oestrogen receptor α (ERα) in normal mammary gland. Staining is restricted to the epithelial cells lining the breast ducts (arrows). (B) Serial section of (A) but stained with a monoclonal antibody directed against ERβ. In addition to staining the ductal epithelium (D), ERβ immunoreactivity is also seen in myoepithelial cells (M) and scattered periductal stromal fibroblasts (F). (C) Breast lobule showing ERβ immunoreactivity in epithelial cells (E), intralobular fibroblasts (F), and in endothelial cells lining a blood vessel (V). (D) Progesterone receptor immunoreactivity in the nuclei of normal breast epithelium (arrow).

appropriate biotinylated secondary antibody (Dako anti-mouse; 1/200 dilution) for 30 minutes at room temperature, then incubation with streptavidin ABC kit (Dako), the ER and PR proteins were visualised with 3,3′-diaminobenzidine (Vector, Peterborough, UK). Negative controls included the omission of the primary antibody or incubation with the appropriate blocking peptide. Sections were lightly counterstained with haematoxylin, dehydrated, and coverslipped. Slides were scored using a system involving the assessment of staining intensity and percentage positivity, which generated a numerical score of 0 to 8.[13] A score of > 2 was classified as positive. Staining was scored independently by two authors

(GPS, PJC). Representative images were captured using a Nikon light microscope attached to a computer equipped with Lucia software (version 4.51).

RESULTS

Using serial sections, the expression and distribution of ERα and ERβ were analysed and compared. In accordance with previous reports, ERα was restricted to the cell nuclei of luminal epithelial cells lining ducts and lobules (fig 1A). However, a much more widespread pattern of staining was seen with ERβ (fig 1B). As with ERα, this receptor was also seen in the nuclei of the same epithelial cells lining breast ducts and lobules (fig 1B). However, additional strong staining was detected specifically in the cell nuclei of myoepithelial cells (fig 1B). Weak to moderate staining was seen in some intralobular stromal cell nuclei, in nuclei of endothelial cells lining blood vessels (fig 1C), and in lymphocytes. Whereas the expression of ERα and ERβ tended to be uniform across a given section, a more patchy distribution pattern was seen for PR, with weak to moderate staining. PR was specifically localised to epithelial cells (fig 1D), and although the coexpression of PR and ERα and ERβ was seen in the same tissue section, it was not always in the same cells.

In general, ERβ immunoreactivity was much stronger than that of ERα, and the staining pattern was identical in breast reduction specimens and in normal tissue adjacent to tumours. In addition, the staining pattern and intensity for ERβ did not appear to be affected by patient age. In all cases, specific staining was abolished in negative controls.

DISCUSSION

We have shown the differential expression of the two ER subtypes—ERα and ERβ—in normal human mammary gland, with much more widespread expression of ERβ compared with ERα.

The distribution patterns of each receptor were distinct. Whereas ERα was restricted to the cell nuclei of epithelial cells, a striking observation was the strong expression of ERβ in the nuclei of myoepithelial cells. The presence of ERβ in these cells may provide clues as to the function of this receptor subtype in the mammary gland. The myoepithelium forms a natural barrier separating proliferating epithelial cells from the basement membrane and the underlying stroma. Myoepithelial cells rarely undergo transformation and there is experimental evidence that they may act as natural tumour suppressors.[14] In the mammary gland, loss of ERβ in the transition from benign lesion to carcinoma in situ has recently been reported,[15 16] prompting the proposal that ERβ may have a role as a tumour suppressor.[15] Strong, specific expression of ERβ in the myoepithelium may strengthen this hypothesis, where it may be fulfilling a protective function. Further evidence in support of this is provided by Taylor and Al-Azzawi,[17] who described increased ERβ immunoreactivity in the glands of normal resting breast tissue compared with proliferative breast tissue.

In gene knockout studies, the presence of ERα but not ERβ is necessary for the development of the mouse mammary gland.[18] Therefore, ERβ may be acting as an antagonist of ERα and removing its antagonistic effect may be akin to "removing a brake". Thus, by removing ERβ the suppressive effect of the receptor is lost. Alternatively, the putative suppressive effects of ERβ may be dictated by the downstream signalling pathway. Both ER subtypes can signal via classic oestrogen response elements or via AP-1 enhancers.[19] The downstream effects of signalling through AP-1 are both receptor and ligand specific. Whereas ERα–17β-estradiol complexes activate gene transcription, the reverse is true for ERβ–17β-estradiol complexes.[20] Thus, if signalling is mediated through AP-1, an inhibitory effect of ERβ might be expected.

Take home messages

- Oestrogen receptor α (ERα) expression was restricted to the cell nuclei of epithelial cells lining ducts and lobules
- ERβ was also expressed in these cells but additional strong staining was seen in the cell nuclei of myoepithelial cells and occasionally in surrounding stromal and endothelial cell nuclei and in lymphocytes
- Thus, ER subtypes have distinct distribution patterns in the normal mammary gland
- The widespread distribution of ERβ suggests that it may be the dominant ER in the mammary gland, where it may be acting as a natural suppressor

ERβ was also observed in lymphocytes, stromal cells, and endothelial cells. It has long been known that oestrogens have important effects on the immune system and to this end ERβ expression in lymphocytes has previously been reported.[3] We and others have reported stromal immunoreactivity for ERβ in the mammary gland,[11 16 17] and this has also been noted in colon[21] and prostate.[22] In the endothelium, where it plays a role in vascular remodelling,[23 24] ERβ is believed to be the dominant receptor subtype. Despite these observations, the functional importance of ERβ in non-epithelial cells is unclear, although it may be fulfilling a paracrine role. Future studies are required to investigate this question.

> "Myoepithelial cells rarely undergo transformation and there is experimental evidence that they may act as natural tumour suppressors"

In the mouse mammary gland, ERβ is expressed by 60–70% of epithelial cells, irrespective of the stage of breast development.[9] In our study, we did not attempt to correlate ERβ expression with the developmental or hormonal state of the mammary gland: all reduction mammoplasty and fibroadenoma samples were from premenopausal patients whose menstrual status was unknown, whereas the normal breast samples adjacent to tumours were predominantly from postmenopausal patients. However, it would be interesting to determine whether the expression of this receptor changes according to the hormonal milieu of the breast. Although age related changes in the expression of ERα have been reported in normal mammary gland, where the proportion of ERα positive cells increases with age,[25] we saw no such changes in either the proportion or degree of expression of ERβ when glands from premenopausal and postmenopausal women were compared.

Although it is well established that ERα is regulated by PR, there have been conflicting reports regarding the relation between ERβ and PR. In the endometrium, PR significantly correlated with ERα, but not with ERβ.[26] A study of human prostate revealed a significant association between PR and both ERα and ERβ, where it was concluded that both ER subtypes could induce PR expression.[27] In our present study, the analysis of serial sections revealed that although ERβ and PR expression could be seen in the same tissue sections, it was not always in the same cells. Thus, it seems likely that there is an association between ERβ and PR in the mammary gland.

In conclusion, ER subtypes have distinct distribution patterns in the normal mammary gland. The widespread distribution of ERβ suggests it may be the dominant ER in the mammary gland, where we propose it may be acting as a natural suppressor of oestrogen activity.

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