Epidermal growth factor receptor and oestrogen receptors in the non-malignant part of the cancerous breast

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Summary Fifty-one samples of non-malignant tissue from four mastectomies were analysed to assess oestrogen receptor (ER) and epidermal growth factor receptor (EGFR) status across the cancerous breast. No significant relationship was found between the presence of EGFR and ER. Eighty-four per cent of these samples were EGFR positive and 29% expressed both receptor types. EGFR and ER expression was not affected by histological sub-group. In contrast, analysis of 44 primary cancers showed, in agreement with the literature, a significant inverse relationship between the presence of ER and EGFR (Fisher’s exact test P<0.002). The difference between malignant and non-malignant tissue appeared to result from the prevalence of co-expression of EGFR and ER in the non-malignant specimens. This suggests different regulation of receptor expression in malignant and non-malignant tissue.

As a consequence of the increasing awareness of the role of peptide growth factors in cellular growth, differentiation and proliferation in normal tissue (Rowe & Friesen, 1984) and in malignancy (Spor & Roberts, 1985; Anonymous, 1986; Lippman et al., 1986, 1987), attention is being focused on the expression of their receptors in breast cancer. The receptor for epidermal growth factor (EGFR) and oestrogen receptor (ER) expression in primary breast carcinomas in that co-expression of these two receptor types is rare, and most tumours are either ER +ve or EGFR +ve (Perez et al., 1984; Sainsbury et al., 1985a, b, 1987, 1988; Skoog et al., 1986; Davidson et al., 1987; Wyss et al., 1987; Macias et al., 1987; Nicholson et al., 1988a, 1989; Pekonen et al., 1988; Travers et al., 1988; Cappelletti et al., 1988; Wrba et al., 1988; Delarue et al., 1988). It has also been suggested that the detection of EGFR in a primary breast tumour indicates a poor prognosis (Sainsbury et al., 1985a, b, 1987, 1988; Nicholson et al., 1989). As the expression of EGFR is not limited to malignant breast cells but has also been found in normal breast tissue adjacent to primary tumours (Ozawa et al., 1988) and in the normal breast cell line, HBL-100 (Fitzpatrick et al., 1984), and furthermore since cultured cells from benign breast fibroadenomas have been shown to respond to EGF in vitro (Stokes et al., 1976), our investigation addresses the incidence of EGFR in the non-malignant part of the cancerous breast and its relationship with oestrogen receptors (ER) and progesterone receptors (PgR).

Forty-four primary breast carcinomas were treated similarly.

Reagents

Radioinert mouse epidermal growth factor (EGF) was obtained from Sigma Chemical Co. (Poole, England). 125I-labelled EGF was produced in our laboratory using iodogen iodination reagent (Pierce UK, Luton), and purified by G-50 Sephadex chromatography, to give 125I-EGF of specific activity of approximately 400 Ci mmol\(^{-1}\). All radioisotopes were purchased from Amersham International plc, England.

Tissue storage and preparation

Specimens were frozen in liquid nitrogen immediately after surgical resection, and stored in liquid nitrogen. Samples were ground in the presence of dry ice in a tissue grinder. Following addition of 10 ml g\(^{-1}\) tissue of 50 mM phosphate buffer (pH 7.4) containing 1.5 mM EDTA, 10% glycerol (all from BDH Ltd., Poole, England), 10 mM monothioglycerol (Sigma), aprotinin (1 µg ml\(^{-1}\); Sigma) and soybean trypsin inhibitor (1 µg ml\(^{-1}\); BDH), further homogenisation was carried out using a Polytron (Kinematica GmbH, Switzerland).

After centrifugation at 100,000 g for 1 h, at 4°C, the supernatant was retained for soluble oestrogen receptor assays. The particulate residue was resuspended in tris buffer (pH 7.4) containing 50 mM sodium chloride and aprotinin and soybean trypsin inhibitor, as above, and retained for EGFR assay.

Protein estimation was carried out using the method of Lowry et al. (1951).

EGF receptor (EGFR) assay

A radioligand binding assay was used to measure EGFR concentrations following the method of Nicholson et al. (1988b). Aliquots of the particulate fraction, representing a crude membrane fraction, were incubated in duplicate with a single concentration of 125I-EGF (final concentration 1 nM). To measure non-specific binding, similar tubes were set up which contained 125I-EGF together with a 100-fold excess of unlabelled EGF (final concentration 100 nM). After a 2-h incubation at 26°C, which was then transferred to an ice-bath and subsequently kept at 4°C. Ice-cold tris buffer (containing 0.1% w/v bovine serum albumin) was then added and the suspension was centrifuged at 10,000 g for 5 min. The supernatant was discarded and the pellet was washed and recentrifuged with a further aliquot of ice-cold tris buffer. This second supernatant was discarded and the pellet was counted in a gamma-counter (NE 1600). Thor Nuclear Enterprises). Rat liver and human placental membrane suspensions were
used as positive controls. Intra-assay variation was less than 5% and inter-assay variation was 9%.

The detection limit for the assay (value significantly different from zero) was 3 fmol mg\(^{-1}\) protein and specific binding was invariably greater than 25% of total binding.

**Steroid hormone receptor radioligand binding assays**

The single-saturating dose assay (King et al., 1979; Puddefoot et al., 1987) was used to measure soluble oestrogen receptors. Briefly, this involved incubation of aliquots of cytosol in the presence of 17 nm tritiated 17-beta oestradiol (sp.act.101 Ci mmol\(^{-1}\)) for 18 h at 4°C, without or with a 100-fold excess of diethylstilboestrol, to determine total and non-specific binding, from which a value for specific (receptor) binding was obtained. Bound radioactivity was separated by the dextran-coated charcoal method and counted by liquid scintillation spectrophotometer. The detection limit for this assay was 5 fmol mg\(^{-1}\) protein.

A single-saturating dose assay also was used to determine progesterone receptor concentrations. The method was as for ER assay except that the radioligand used was tritiated progesterone (sp.act. 85 Ci mmol\(^{-1}\)) at a final concentration of 28 nM.

A 100-fold excess of 19-norethindrone was used to estimate non-specific binding and all tubes contained cortisol (final concentration 1 x 10\(^{-6}\)M) to eliminate binding to corticosteroid binding globulin. The detection limit for this assay was 10 fmol mg\(^{-1}\) protein.

**Histopathology**

Samples fixed in buffered formalin were processed to paraffin in the usual way and 5 μm sections examined in haematoxylin and eosin preparations (Panahy et al., 1987).

**Results**

**Non-malignant tissue**

Sixty-two specimens from the four 16-sector mastectomies were analysed. However, as malignant cells were found in 11 of these, only 51 histologically proven non-malignant specimens were included in this group. Of these, 84% (43/51) were EGFR positive and of the ER positive samples only 12% (2/17) were EGFR negative. Figure 1 shows the concentration and distribution of ER and EGFR in the non-malignant group. EGFR positive concentrations ranged from 3 to 39 fmol mg\(^{-1}\) protein; ER positive from 5 to 134 fmol mg\(^{-1}\) protein. No significant relationship was found between the presence of ER and EGFR in the non-malignant group using either Fisher’s exact test (P<0.30) or Kendall’s rank correlation (P<0.2).

The non-malignant samples could be divided, according to the predominant tissue present, into three different histological sub-groups of normal/fat, fibrous/connective and benign/fibrocystic tissue. Figure 2 shows their respective receptor concentration ranges. No significant differences were found between the means of the positive values in each sub-group with respect to either ER concentrations or EGFR concentrations, or when comparing receptor concentrations between the four individual mastectomies (Table I). Similarly, no significant differences were found between the relative proportions of each receptor phenotype in a given sub-group (Table II). Table II also includes data on mastectomy samples with tumour infiltration. Furthermore, no significant differences in receptor phenotype distributions were found between samples taken from the two premenopausal and the two post-menopausal patients, and no relationships were found with respect to the anatomical segments from which the samples were obtained in any of the individual patients. PgR concentrations where positive ranged from 10 to 500 fmol mg\(^{-1}\) protein. No significant relationships were found between PgR and either ER or EGFR expression.

Three samples of breast tissue were also obtained from one patient undergoing reduction mammoplasty. Of these, all were EGFR positive (4–14 fmol mg\(^{-1}\) protein) and one was also found to be positive for ER (30 fmol mg\(^{-1}\) protein).

**Primary breast cancers**

In contrast to the non-malignant group, of 44 primary breast cancers analysed, 71% (32/44) could be classified as either ER+/EGFR- or ER-/EGFR+. Figure 3 shows the receptor concentrations of ER and EGFR in this series of tumour samples. EGFR positive concentrations ranged from 3 to 57 fmol mg\(^{-1}\) protein; ER positive from 5 to 77 fmol mg\(^{-1}\) protein. A highly significant inverse relationship was shown to exist between ER and EGFR expression in the malignant group, so that in ER+ve primary cancers EGFR were usually undetectable, and vice versa (Fisher’s exact test, P<0.002).

Figure 4 compares distribution of receptor phenotypes in the non-malignant and malignant groups. Of the ER positive non-malignant samples 88% (15/17) were EGFR positive.
Table I  Analysis of data from individual patients comprising the non-malignant tissue group

| Patient | Receptor concentration (fmol mg⁻¹ protein) | ER | Median | EGFR | Median |
|---------|--------------------------------------|-----|--------|------|--------|
| P.D.    | 5-32                                 | <5  | 3-21   | 6.2±4.7 | 4     |
| C.S.    | 5-18                                 | <5  | 3-23   | 7.5±5.7 | 5     |
| M.P.    | 5-40                                 | <5  | 3-39   | 12±11.41| 6.5   |
| C.G.    | 5-134                                | <5  | 3-9    | 6.2±2   | 5     |

Patients P.D. and C.S. were premenopausal and patients M.P. and C.G. were post-menopausal.

Table II  Distribution of receptor phenotypes in each histological sub-group of non-malignant cancerous breast

| Receptor phenotype | EGFR + / ER- | EGFR + / ER + | EGFR- / ER- | EGFR- / ER + |
|--------------------|---------------|---------------|-------------|--------------|
| Normal/fat         | 10 (62.5)     | 4 (25)        | 2 (12.5)    | 0            |
| Fibrous/connective | 5 (62.5)      | 2 (25)        | 1 (12.5)    | 0            |
| Benign/fibrocystic | 13 (49)       | 9 (33)        | 3 (11)      | 2 (7)        |
| Tumour infiltration| 6 (55)        | 2 (18)        | 3 (27)      | 0            |

Percentage of samples with each receptor phenotype for a particular sub-group in parentheses.

Figure 3  Pooled data showing receptor distribution in a series of 44 primary breast cancers. EGFR concentration measured by radioligand binding assay (y axis). ER concentration measured by radioligand binding assay (x axis). Dashed lines indicate detection limits of each assay. Points to the left of the vertical dashed line represent samples in which ER concentrations were undetectable. Points below the horizontal dashed line represent undetectable EGFR.

while only 17% (3/23) of the ER positive tumours also expressed EGFR. In addition, whether ER + or ER −, the proportion of EGFR + ve samples was greater in the non-malignant group; 43/51 (84%) versus 15/44 (34%).

PgR concentrations where positive ranged from 10 to 526 fmol mg⁻¹ protein and no significant relationships were found between PgR and either ER or EGFR in the tumour group.

Comparison of tissue yields between non-malignant tissue groups and the tumour group (Table III) indicates that the latter shows relatively higher protein yields for both membrane and cytosolic fractions.

Discussion

Our data represent the first systematic study of the relationship between EGFR and ER in the cancerous breast after excision of the primary cancer. EGFR is present in all types of non-malignant tissue although its level of expression may be relatively low compared with the top range previously reported in primary breast tumours. Furthermore, the results from the series of tumours analysed in this study agree with previous work in demonstrating the absence of EGFR and ER co-expression, and it is therefore extremely significant to find that in the non-malignant group the two receptor types

![Graph showing receptor distribution](image-url)

Figure 4  Comparison of receptor phenotype distribution in tumour and non-malignant samples. Height of bars represents percentage of total samples expressing a given phenotype.

Table III  Comparison of tissue yields from the different histological groups

| Histological group | Membrane protein | Cytosolic protein |
|--------------------|------------------|-------------------|
| Non-malignant      | 2.1 ± 1.8        | 16.2 ± 8.0        |
| Normal/fat         | 1.4 ± 1.1        | 14.7 ± 7.4        |
| Fibrous/connective | 0.8 ± 1.0        | 10.4 ± 7.0        |
| Benign/fibrocystic | 2.8 ± 2.0        | 18.8 ± 7.8        |
| Tumour infiltration| 3.8 ± 3.2        | 28 ± 10           |
| Primary tumours    | 3.7 ± 2.4        | 26.1 ± 7.8        |

Samples in the non-malignant group were obtained from four individual mastectomies. The primary tumours were taken from 44 individual patients.
were found to co-exist in a significant proportion of specimens analysed, irrespective of histological sub-group. This finding is the more remarkable in view of the highly heterogeneous nature and the frequently low cellularity of non-malignant breast tissue (Table III). The results suggest that the inverse relationship between ER and EGFR which is found in tumours may reflect an abnormal regulatory state confined to the tumour itself. Travers et al. (1988) have also analysed non-malignant breast tissue for the presence of growth factor mRNA. Their results indicate that, while an inverse relationship between ER-mRNA and EGFR-mRNA could be found in malignant tumours, EGFR-mRNA was always detectable in benign tumours and in four samples of normal breast, although in one case. Our results confirm and extend these findings to receptor protein expression in non-malignant tissue throughout the cancerous breast.

One explanation for the differences observed between tumour and non-malignant breast tissue with respect to ER and EGFR could be that during the process of malignant transformation tumour cells acquire the ability to synthesise transforming growth factor alpha (TGF-alpha), a known mitogen which acts via EGFR (Stoschek & Lloyd, 1986). It might be argued that if TGF-alpha is produced in tumours in sufficiently high concentration (Dickson & Lippmann, 1986; Bates et al., 1988) EGFR sites may be saturated and thus be undetectable by radioligand binding assay, whereas in normal tissue low levels of EGFR might be detectable. This argument would agree with the observations of Travers et al. (1988), who showed that mRNA for ER and EGFR were not found to co-exist to any significant extent in primary breast carcinomas. It is well established that in addition to stimulating EGFR mRNA synthesis, EGFR or TGF-alpha can cause 'down-regulation' of EGFR by receptor internalisation and degradation (Carpenter, 1987; Schlessinger, 1988). It might be, therefore, that long-term exposure to TGF-alpha leads to suppression of EGFR mRNA synthesis.

Sainsbury et al. (1987) and Nicholson et al. (1988b, 1989) have suggested a clinically significant cut-off value for EGFR in tumours of 10 fmol mg^-1 protein. Using this limit they have shown that the presence of an EGFR positive tumour indicates a worse prognosis, in terms of relapse-free survival, compared with EGFR negative tumours. However, it is clear from our data that the observations of a tumour with EGFR + ve/ER - ve phenotype may not necessarily be abnormal as 55% of our non-malignant samples expressed this phenotype and only one primary tumour had an EGFR concentration above the range of concentrations found in the non-malignant group. Ozawa et al. (1988) have suggested that EGFR binding is significantly higher in breast tumours compared to normal adjacent tissue, although 50% of their tumours gave EGFR binding levels within the range of the normal samples. It is likely therefore that the significance of EGFR as a prognostic indicator is the result of a number of factors. There may be an advantage conferred to the EGFR negative group by the presence of a functional ER system, but a disadvantage associated with over-expression of EGFR arising from the EGFR and ER pathways, therefore, that have shown in non-malignant tissue both EGFR and ER can more often be detected in the Travers et al. (1988) study, implying that normally both receptor types are expressed.

We conclude therefore, from our data on non-malignant tissue, that in the normal breast both ER and EGFR co-exist to maintain regulated cell growth, and that this system becomes uncoupled during malignant transformation.

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