Pergamon

European Journal of Cancer Vol. 31A, No. 12, pp. 2049–2052, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00430-0

Original Paper

Multistep Progression from an Oestrogen-dependent Growth Towards an Autonomous Growth in Breast Carcinogenesis

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We investigated the relationship between hormone receptor status and cellular proliferation in a series of proliferative epithelial breast lesions in an attempt to clarify the putative role of this interaction in the process of breast carcinogenesis. The separation of oestrogen receptor (ER) positive and ER negative cases revealed that in hyperplastic breast epithelium (with and without atypia) the ER positive cases had a higher proliferation rate than that of ER negative cases. Conversely, in ductal carcinomas (both *in situ* and invasive), ER negative cases had rates of proliferation higher than those observed among the ER positive cases. The observation of higher proliferation in ER positive benign proliferative breast lesions fits with the concept of an initial hormone-dependent status in breast carcinogenesis. According to this assumption, activation of ER by hormone increases the possibility that cells may undergo malignant transformation. Although we are limited by our static view of the process, our results point to the existence of successive steps of progression from a hormone-dependent towards an autonomous growth. The demonstration of higher proliferation in ER-negative carcinomas, from the *in situ* phase onwards, reinforces the hypothesis that breast cancer progression is paralleled by a progressive hormone independence.

Key words: breast carcinogenesis, ER content, cell proliferation
Eur J Cancer, Vol. 31A, No. 12, pp. 2049–2052, 1995

INTRODUCTION

OESTROGENS PLAY an important role in the normal physiology of the breast and have a well-established proliferative effect upon breast tissue [1]. The clear relationship between oestrogens and the breast has led many investigators to suggest that these hormones are likely to contribute to tumour formation in mammary tissue. This assertion is strengthened by the finding that the oestrogen receptor (ER), the cellular target for oestrogens, serves as a hormone-dependent transcription factor that helps to regulate the expression of a variety of autocrine and paracrine growth factors in breast tumour cell lines [2].

Recently, it was suggested that ER positivity of benign breast epithelium could be a risk factor for breast malignancy [3], because the presence of ER is thought to render cells susceptible to the proliferation stimulus of oestrogens. The increasing number of mitotic events could provide opportunities for genomic instability and initiation of malignancy during cell division. Considering that: (a) the exposure of the terminal duct-lobular unit of the breast to oestrogens seems to be a critical event on mammary carcinogenesis [4, 5]; (b) ER-positivity of this epithelium is associated with a higher incidence of breast cancer [3]; and (c) there is clinical, epidemiological and experimental evidence showing that ductal hyperplasia represents a step of the progression towards the development of breast carcinomas [5, 6], we decided to study the relationship between hormone receptor status and cellular proliferation in a series of proliferative epithelial breast lesions in an attempt to clarify the putative role of this interaction in the process of breast carcinogenesis.

Revised 21 Apr. 1995; accepted 1 May 1995.

MATERIALS AND METHODS

Specimens

Routinely formalin-fixed, paraffin-embedded breast tissue from 40 patients with intraductal epithelial proliferations (mean age 41, range 27–63 years) and 40 with invasive carcinomas (mean age 54, range 33–77 years) were investigated. We also included 20 cases of normal breast tissue obtained from plastic surgery procedures. The spectrum of histological intraductal proliferations was subdivided into 20 cases of ductal hyperplasia without atypia; 5 cases of atypical ductal hyperplasia; and 15 cases of ductal carcinoma *in situ* (DCIS). The criteria for classifying the hyperplasias as typical or atypical were those described by Page and Rogers [7]. The DCIS were classified

according to the predominant architectural pattern into: comedo (n = 8), cribriform (n = 3) and micropapillary (n = 4) following the criteria of Page and Anderson [8]. All invasive carcinomas studied were infiltrating ductal carcinomas classified according to WHO recommendations [9].

Tissues from all cases had been fixed in buffered 10% formalin, dehydrated and embedded in paraffin; 5 μm sections were cut and stained with haematoxylin and eosin and used to classify all the lesions.

Immunohistochemistry

For immunostaining, the avidin–biotin–peroxidase complex (ABC) method was used. Briefly, the sections from formalin-fixed material were cut, dewaxed and then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to quench the endogenous peroxidase activity. The slides were then briefly rinsed in distilled water and incubated for 10 min at 750 W (domestic microwave) in 10 mM citrate buffer in a thermoresistant container. Distilled water and buffer were added periodically to the container to prevent drying during the incubation process. The slides were cooled in buffer for 20 min (to room temperature), washed in distilled water, and rinsed in phosphate-buffered saline (PBS). The primary antibodies (see below) were applied to the sections and incubated overnight at 4°C. This was followed by incubation with a 1:100 dilution of biotin-labelled anti-mouse secondary antibody (Dakopatts, Copenhagen, Denmark) for 30 min and ABC (Dakopatts) for 60 min. Careful rinses were done with PBS between each step of the procedure. The colour was developed with diaminobenzidine and the sections were lightly counterstained with haematoxylin, dehydrated and mounted.

The primary antibodies used were: MIB 1 (Immunotech S.A., Marseille, France) diluted 1:100 and oestrogen receptor clone ER1D5 (Dakopatts) diluted 1:200. Negative controls for the immunostaining were carried out by substituting the primary antibody with a mouse myeloma protein of the same subclass and concentration of this primary antibody. As a positive control, sections from cases of invasive breast carcinoma known to express MIB-1 and ER were used.

Evaluation of immunohistochemical data

MIB 1 immunostaining (MIB 1LI : MIB 1 Labeling Index) was scored by counting 1000 cells in 10–20 fields per histological section depending on cellularity. Every stained nucleus was considered positive regardless of the intensity of the staining. In cases where there was a clear cut variability of stained cells in different areas of the section, the fields examined included those with the highest and those with the lowest percentage of stained cells.

The immunostaining for ER was assessed on the basis of the visually estimated percentage of neoplastic cells with positive nuclear staining. Scoring was made in a minimum of 500 cells by counting the intensity and percentage of cells with nuclear staining for ER, and expressing this as an H-score between 0 and 300. Positive cases were defined as having an H-score greater than 20. We have previously described the excellent agreement between the ER assessment in formalin-fixed material and frozen sections [10].

Statistical analysis

Statistical analysis was performed using the Statview programme on an Apple Macintosh LC III computer. The values are presented in percentages or means ± standard error (S.E.). Statistical differences between mean values of the groups were evaluated using Analysis of Variance (ANOVA). P values of < 0.05 were considered significant.

RESULTS

ER content

ER immunostaining was localised in the nuclei and showed some variability in intensity. The ER positivity rate was similar for each type of lesions: ductal hyperplasia without atypia (75.0%), atypical hyperplasia (60.0%), ductal carcinoma *in situ* (60.0%) and invasive carcinoma (62.5%) (Table 1).

MIB 1 immunostaining

MIB 1 staining was confined to nuclei with diffuse staining pattern. The intensity of staining varied from lesion to lesion, presumably reflecting not only inherent differences between the lesions, but also minor differences in specimen handling. There was also some variation in staining among the nuclei of each tumour.

The MIB 1 LI was significantly higher ($P < 0.001$) in both types of malignant lesions (DCIS: 18.3% and invasive carcinomas: 20.8%) than in the benign lesions (ductal hyperplasia without atypia: 7.9% and atypical hyperplasia: 7.2%) (Table 1).

Association between ER content and MIB 1 LI

The correlation between ER and MIB 1 immunostaining in the different types of breast lesions is summarised in Table 2. The separation of ER positive and ER negative cases revealed that in hyperplastic breast epithelium (with or without atypia) the ER positive cases had a higher mean MIB 1 LI than that of ER negative cases (Table 2). Conversely, in ductal carcinomas (both *in situ* and invasive), ER negative cases had rates of proliferation higher than those observed among the ER positive cases (Table 2).

DISCUSSION

There are few studies which have focused on the receptor content and proliferation rates of non-malignant breast tissues because production of highly specific monoclonal antibodies to ER and Ki-67, that work well in routinely processed material, is rather recent [11, 12]. Our results are similar to those described by other authors for ER [3, 13], and proliferative rates [14] in non-malignant breast epithelium. However, these studies did

Table 1. Oestrogen receptor (ER) content and MIB-1 labelling index (MIB-1 LI) in different types of breast lesions

Histological diagnosis	ER positivity n (%)	MIB-1 LI (mean ± S.E.) (range)
Normal breast (n = 20)	16 (80.0)	3.7 ± 0.7 (0.0–12.0)
Ductal hyperplasia without atypia (n = 20)	15 (75.0)	7.9 ± 0.4 (4.9–15.0)
Atypical hyperplasia (n = 5)	3 (60.0)	7.2 ± 1.1 (5.4–12.5)
Ductal carcinoma *in situ* (n = 15)	9 (60.0)	18.3 ± 1.1 (10.0–26.0)
Invasive carcinoma (n = 40)	25 (62.5)	20.8 ± 2.7 (5.0–90.0)

Table 2. Comparison of the MIB-1 labelling index (MIB-1 LI) between oestrogen receptor (ER) positive and ER negative cases within each category of breast lesions

Histological diagnosis	MIB-1 LI (mean ± S.E.) (range) ER positive	ER negative	*P* value
Normal breast (n = 20)	3.9 ± 0.8 (0.0–12.0)	3.0 ± 1.2 (0.0–6.0)	0.62
Ductal hyperplasia without atypia (n = 20)	10.2 ± 0.5 (7.0–15.0)	6.5 ± 0.7 (4.9–9.0)	0.003
Atypical hyperplasia (n = 5)	8.0 ± 2.0 (5.4–12.5)	6.0 ± 0.2 (5.8–6.2)	0.74
Ductal carcinoma *in situ* (n = 15)	13.4 ± 0.9 (10.0–18.0)	20.6 ± 1.2 (17.1–26.0)	0.001
Invasive carcinoma (n = 40)	14.6 ± 1.3 (6.2–30.7)	31.2 ± 24.6 (5.0–90.0)	0.003

not try to correlate ER and proliferation in the different types of lesions that putatively precede breast carcinoma.

Assuming that, at least, some breast carcinomas arise through a series of progressively abnormal cellular changes, ranging from hyperplasia to malignant invasive neoplasia [5], we can, from a practical viewpoint, study the different steps of breast carcinogenesis. Our findings support the assumption that hormonal stimuli that induce growth and differentiation in the normal breast also contribute to the development of mammary malignancy [3, 4]. Oestrogen hormones are considered to be promoters of initiated breast cancer cells, and to act over many years to increase the populations of initiated cells. Recently, it was demonstrated that sex steroid hormones, including oestrogens, exert effects on cell proliferation by affecting cell cycle control points in the G1 phase [15]. Oestrogens increase the rate of cell proliferation by both recruiting non-cycling cells into the cell cycle and by shortening the overall cell cycle time due to a reduction in the length of the G1 phase [1].

The demonstration that some nuclear proto-oncogenes, namely *C-FOS*, *C-JUN* and *C-MYC*, and G1 cyclins, that have key regulatory roles in cell cycle control, are transcriptionally regulated by steroids, has led many researchers to suggest that these hormones are likely to contribute to carcinogenesis in breast tissue [2, 15, 16].

The observation of higher rates of proliferation in ER positive benign proliferative breast lesions fits with the concept of an initial hormone-dependent status in breast carcinogenesis. According to this assumption, activation of ER by the hormone increases the possibility of cells undergoing malignant transformation. Stimulation of the mitotic activity of breast cells caused by cyclic release of oestrogens during the menstrual cycle or by an exposure to exogenous oestrogens enhances the opportunity for occurrence of mutations and other replicative errors associated with DNA synthesis and cell division [16].

Although limited by the static view of the process, our results point to the existence of successive steps of progression from a hormone-dependent towards an autonomous growth. The demonstration of higher proliferation rates in ER negative carcinomas, from the *in situ* phase onwards, reinforces the hypothesis that breast cancer progression is paralleled by a progressive hormone independence. The inverse relationship between hormonal status and expression of several growth factors and their receptors (including overexpression of C-ERBB-2) reported in other series [17, 18] supports this assumption. However, the absence of ER immunoreactivity in several cases of breast carcinoma does not imply a true ER negativity since there is a significant body of evidence that many human breast tumours produce defective forms of ER [16].

Rearrangements or deletions in the steroid receptor genes or their mRNAs that cause premature termination of translation, can produce receptors that stimulate the expression of steroid-responsive genes even in the absence of hormone. This implies that breast cancer cells that express ER variants due to a genetic or epigenetic change will begin to proliferate as if they were chronically stimulated by oestrogen. Moreover, ER variants cannot be detected by current clinical assays to measure ER, thus limiting the diagnostic and predictive value of these tests [16].

Loss of ER expression as detected by the usual assays, such as ligand-binding and immunohistochemistry, can occur in two ways. Somatic mutations in the ER gene have been described in sporadic breast tumours, but this appears to be an unusual finding and an infrequent cause of receptor variants [16, 19]. For reasons that remain unclear, suppression of transcription of the ER gene frequently occurs without involving deletion or rearrangement of the ER gene. In these cases, the gene remains transcriptionally active and gives rise to a number of inactive or dominant inhibitory variants of ER, probably reflecting aberrant RNA processing. Dominant inhibitory mutants may contribute to the emergence of hormone independence and tamoxifen resistance [16, 19]. By recruiting wild-type receptors into inactive heterodimers and thereby blocking their activity, dominant inhibitory mutants may intensify selective pressures, favouring the outgrowth of cells that contain weakly constitutive mutants of ER. This may represent a transitional state in the progression of ER positive cells to hormone independence. Properties such as increased proliferation, hormone independence, and tamoxifen resistance may result directly from the emergence of constitutive ER variants. These events are commonly regarded as pivotal steps in the malignant progression of breast cancer. Although recognising that it is far beyond the scope of this study to detect structural variants of ER, we think we have found enough evidence, using the current methods of demonstrating ER expression and evaluating proliferative indices, to claim that our results fit with the aforementioned possibility.

In conclusion, we have demonstrated the existence of a positive correlation between ER status and proliferation in ductal hyperplasias and a progressive inversion of this relationship in lesions evolving towards malignancy. According to our results, the anti-oestrogenic approach could represent an appropriate strategy for primary prevention of breast cancer, diminishing the proliferative rates of early hyperplastic lesions. However, this assumption needs to be proved in large clinical trials.

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Acknowledgements—We wish to thank Professor Manuel Sobrinho-Simões (Unit of Molecular Pathology, IPATIMUP, Medical Faculty, Hospital S. João, Porto) for his helpful advice in the preparation and revision of the manuscript. This work was partially supported by a research grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (No. 201240/92).