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Primary research

In vivo cell kinetics in breast carcinogenesis

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Abstract

Background: Disruption of the balance between apoptosis and proliferation is considered to be an important factor in the development and progression of tumours. In the present study we determined the *in vivo* cell kinetics along the spectrum of apparently normal epithelium, hyperplasia, preinvasive lesions and invasive carcinoma, in breast tissues affected by fibrocystic changes in which preinvasive and/or invasive lesions developed, as a model of breast carcinogenesis.

Methods: A total of 32 areas of apparently normal epithelium and 135 ductal proliferative and neoplastic lesions were studied. More than one epithelial lesion per case were analyzed. The apoptotic index (AI) and the proliferative index (PI) were expressed as the percentage of TdT-mediated dUTP-nick end-labelling (TUNEL) and Ki-67-positive cells, respectively. The PI/AI (P/A index) was calculated for each case.

Results: The AIs and PIs were significantly higher in hyperplasia than in apparently normal epithelium ($P=0.04$ and $P=0.0005$, respectively), in atypical hyperplasia than in hyperplasia ($P=0.01$ and $P=0.04$, respectively) and in invasive carcinoma than in *in situ* carcinoma ($P<0.001$ and $P<0.001$, respectively). The two indices were similar in atypical hyperplasia and in *in situ* carcinoma. The P/A index increased significantly from normal epithelium to hyperplasia ($P=0.01$) and from preinvasive lesions to invasive carcinoma ($P=0.04$) whereas it was decreased (non-significantly) from hyperplasia to preinvasive lesions. A strong positive correlation between the AIs and the PIs was found ($r=0.83$, $P<0.001$).

Conclusion: These findings suggest accelerating cell turnover along the continuum of breast carcinogenesis. Atypical hyperplasias and *in situ* carcinomas might be kinetically similar lesions. In the transition from normal epithelium to hyperplasia and from preinvasive lesions to invasive carcinoma the net growth of epithelial cells results from a growth imbalance in favour of proliferation. In the transition from hyperplasia to preinvasive lesions there is an imbalance in favour of apoptosis.

Keywords: apoptosis, breast carcinoma, hyperplasia, proliferation

Introduction

Tissue homeostasis is maintained through a balance between cell proliferation and apoptosis. Both are active

processes that are influenced by a wide variety of regulatory stimuli. Each cell is under constant surveillance in order to maintain the integrity of its genome. When onco-

genic mutations occur, potent mechanisms exist that limit the expansion of affected cells by suppressing their proliferation or triggering their apoptosis [1–4].

For some time the deregulation of growth that leads to cancer was explained largely in terms of increased cell proliferation. However, during the past decade the role of apoptosis in tumourigenesis has been extensively studied. Reduced apoptosis may lead to a shift in tissue kinetics toward the expansion of cell numbers, and to the preservation of genetically aberrant cells, favouring clonal expansion and neoplastic development [2,5]. Investigators are increasingly describing cancer as a disease that involves both excessive cell proliferation and inhibition of the cell's ability to die.

Data from histological, epidemiological and experimental studies suggest that breast carcinogenesis is a multistep process that starts with hyperplasia, progressing through atypical hyperplasia to *in situ* and invasive carcinoma. The time course of these changes is difficult to estimate because, during this multistep process, unknown factors may halt progression through the continuum, and the hyperplastic lesions may regress and never undergo a malignant transformation [6–10]. There is no general consensus that the multistep model of breast carcinogenesis is accurate. Different disease progression models have been proposed and studied [11–14]. *De novo* breast carcinogenesis cannot be ruled out.

The role of cell kinetics in development and progression of breast carcinoma remains to be elucidated. Allan *et al* [15] found higher mitotic and apoptotic activities in breast carcinoma than in normal epithelium; they also found and reduced apoptosis and apoptosis relative to mitosis in apparently normal epithelium from breasts with fibrocystic changes and carcinoma, as compared with breasts with fibroadenomas. In another morphological study [11], two disease progression models were used: the well differentiated and poorly differentiated models. In that study, it was found that the progression from well-differentiated preinvasive lesions to well-differentiated invasive carcinoma is accompanied by increased mitotic index, whereas in poorly differentiated lesions decreased apoptosis appears also to be important. Harn *et al* [16] reported a higher degree of TUNEL index and lower proliferative activity in intraductal than in invasive ductal carcinomas.

In the present study, we determined the incidence of apoptosis (detected using the TUNEL method) and cell proliferation (assessed using the Ki-67 antibody), and the balance between these cellular events in apparently normal epithelium, hyperplasia, atypical hyperplasia, and *in situ* and invasive carcinomas, as a model of disease progression. Both AI and PI were evaluated in at least two ductal lesions per case, because the aim of this study was

to study the *in vivo* cell turnover in breast tissue with fibrocytic changes, in which preinvasive lesions and/or invasive carcinoma developed.

Materials and methods

Case selection

Formalin-fixed, paraffin-embedded tissue samples from 67 patients (45 obtained from modified radical mastectomies and 22 from lumpectomies) were used. The patient's ages ranged from 38–73 years (median age 49 years). Eleven patients were premenopausal, and the rest were peri- or postmenopausal. None of the patients was treated before removal of the tumour. Specimens were selected from the files of the Department of Pathology of the University Hospital of Ioannina, Greece. All of the hematoxylin and eosin-stained slides were reviewed, independently, by two pathologists. Only samples from patients in whom more than one lesion was detected were included in the study. Two to four slides per case were selected because each lesion was studied in a different area. The corresponding tissue blocks were then used for *in situ* end-labelling (TUNEL) to detect apoptosis, and for Ki-67 immunostaining to detect cell proliferation. Apoptotic and proliferative activity were evaluated in identical areas in serial sections.

The criteria of Page and Rogers [17] were used to identify hyperplasia of usual type and atypical hyperplasia. Invasive carcinomas were classified according to the criteria of Bloom and Richardson, as modified by Elson and Ellis [18], and *in situ* ductal carcinomas according to the criteria of Holland *et al* [19].

A total of 32 samples of apparently normal epithelium and 135 epithelial proliferative lesions were studied. The lesions comprised the following: 43 ductal hyperplasias of usual type (15 mild, 10 moderate, 18 severe); 17 atypical ductal hyperplasias; 25 *in situ* ductal carcinomas (6 low grade, 8 intermediate, 11 high); and 50 infiltrating ductal carcinomas (12 grade I, 20 grade II, 18 grade III). In 12 out of 25 *in situ* carcinomas an invasive component coexisted, and in 9 out of 15 atypical hyperplasias an *in situ* and/or invasive carcinoma was detected.

TdT-mediated dUTP nick end-labelling assay

The *in situ* visualization of DNA fragmentation was performed on paraffin tissue sections (4 µm thick) placed on superfrost slides, using the commercial *in situ* end-labelling Apop Tag Peroxidase and Fluorescein Kit (Oncor; Gaithersburg, MD, USA). After deparaffinization and dehydration, slides were rinsed in phosphate-buffered saline (pH 7.2). Tissue sections were then digested by incubation for 20 min with proteinase K (20 µg/ml; Oncor) at room temperature, and then were rinsed in distilled water. The peroxidase activity was blocked by incubation for 5 min in 2% hydrogen peroxide in

phosphate-buffered saline. After the application of an equilibration buffer, slides were incubated in working strength TdT enzymes that contained dUTP-digoxigenin for 1 h at 37°C. The reaction was stopped by a pre-warmed (37°C) working strength stop/wash buffer, and then slides were rinsed in phosphate-buffered saline. The incorporated nucleotides were identified by adding peroxidase-conjugated or fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin antibody. Slides visualized by antidigoxigenin-peroxidase were incubated with 3,3'-diaminobenzidine tetrahydrochloride (Oncor) and counterstained lightly with Harris' haematoxylin. Slides visualized by antidigoxigenin-FITC were counterstained with propidium iodide (2.5 µg/ml; Oncor). Negative control slides were prepared by omitting the TdT reaction step. These sections did not show any nonspecific reaction. Apoptosis within the germinal centres of reactive lymph nodes served as positive controls.

Immunohistochemistry

Immunohistochemical staining for Ki-67 antigen was performed on paraffin sections (4 µm thick) placed on poly-L-lysine-coated glass slides. Polyclonal rabbit antibody to human Ki-67 protein was obtained from Dako (A047; Dako SA, Glostrup, Denmark). This antibody has a reactivity similar to that seen with the monoclonal antibody anti-Ki-67 [12]. After deparaffinization and dehydration, sections were treated twice for 5 min in citrate buffer (0.01 mol/l, pH 6.0) in a microwave oven at 700 W. The slides were then cooled to room temperature for 1 h. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in methanol for 30 min. Nonspecific binding of the secondary antibody was blocked by incubating the slides with normal rabbit serum (1/50 dilution) for 1 h. Sections were then incubated with primary rabbit anti-human Ki-67 antibody (1/100 dilution) for 1 h at room temperature. Biotinylated goat anti-rabbit secondary antibody was applied for 60 min at room temperature. Bound antibody was visualized with avidin-biotin-peroxidase complex (Dako Duet Kit; Dako, K0492; 1/200 dilution) for 1 h at room temperature. The colour was developed by 3,3'-diaminobenzidine tetrahydrochloride. Between steps, the slides were rinsed three times for 10 min in tris-buffered saline (pH 7.6). The slides were counterstained lightly in Harris' haematoxylin, and were then dehydrated and mounted. Positive control slides were included in all tests, and consisted of paraffin sections from lymph nodes with follicular hyperplasia. Negative control slides were prepared by omitting the primary antibody.

Counting procedure and assessment of apoptotic and proliferative indices

For the assessment of TUNEL- and Ki-67-positive cells, light microscopic examination was performed at a magnification of ×400 with the use of a counting grid. Because

the rate of apoptosis was low, TUNEL-positive nuclei were counted in each selected slide and at least 3000–4000 epithelial cells were evaluated. The AI (expressed as a percentage) was determined from the percentage of the ratio of TUNEL-positive cells to the total number of cells counted. Areas of obvious tissue necrosis were excluded from counting. The positive nuclei in Ki-67 staining were counted in at least 3000 epithelial cells. The percentage of the ratio of Ki-67 positive cells (PI, also expressed as a percentage) was calculated. All sections were independently evaluated by the two first authors, in collaboration with other members of the staff.

Statistical analysis

For the statistical evaluation of the results, we used Statistica for Windows (97 edition; Microsoft Corporation), run on an IBM-compatible personal computer. Differences among the five tissue groups were assessed by Kruskal-Wallis analysis of variance. Mann-Whitney test was used to compare AI, PI and P/A index in the breast lesions. Correlations between the various indices were identified using Spearman's rank test. All values are expressed as medians (range). $P < 0.05$ was considered statistically significant.

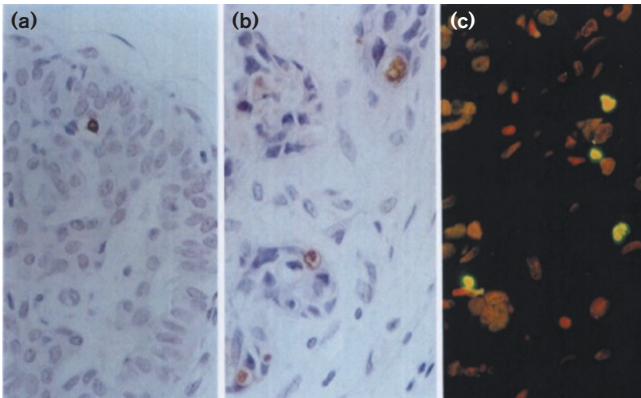
Results

Apoptotic and proliferative indices

The AI, as assessed using the TUNEL method, was evaluated in all cases. Cells with and without morphological criteria of apoptosis (shrinkage of the cytoplasm, condensation and fragmentation of the nuclear chromatin – membrane blebbing) and apoptotic bodies were positive (Fig. 1a–c). Nonlabelled cells that satisfied morphological criteria of apoptosis were counted as apoptotic cells. TUNEL-positive apoptotic cells were easily distinguished from necrotic cells, because they were isolated. In order to avoid false-positive results in the detection of apoptotic cells, the haematoxylin and eosin consecutive stained sections and TUNEL sections were compared. Reactive lymphocytes around ducts or nests of neoplastic cells were rarely TUNEL positive. Negative controls run in the TUNEL assay showed no such reactivity. The AIs obtained using peroxidase- and FITC-conjugated antidigoxigenin antibody were not significantly different ($P = 0.17$).

The PI, as assessed using Ki-67 immunostaining, was evaluated in all cases. Staining intensity varied considerably among nuclei. In *in situ* and invasive breast carcinomas, as well as in lymphoid germinal centres of reactive lymph nodes (used as positive controls), in which the AIs and PIs were higher, we observed Ki-67-positive and negative cells that satisfied morphological criteria of apoptosis. Both Ki-67-positive and -negative cells were at different stages of the apoptotic process (Fig. 2a, b). There were also Ki-67-positive and -negative apoptotic bodies (Fig. 2c).

Figure 1



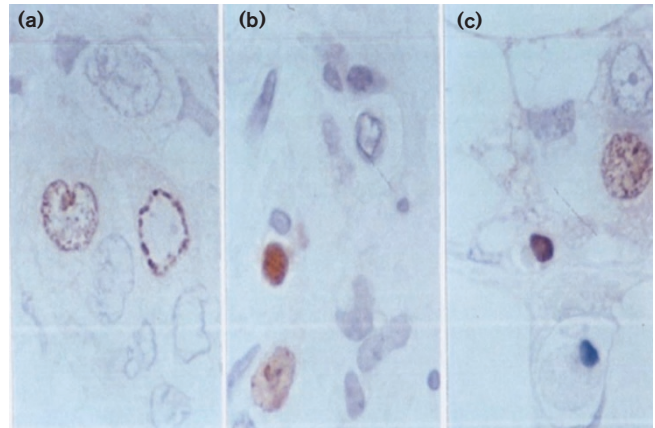
Apoptotic cells in **(a)** ductal hyperplasia and **(b, c)** invasive ductal nonspecific-type carcinomas detected using the TUNEL method. **(a, b)** Antidigoxigenin-peroxidase and **(c)** antidigoxigenin-FITC visualization; 400× each.

The AIs and PIs for each tissue group of lesions are summarised in Table 1. It was obvious from the median values that both AI and PI increased with the progression of epithelial lesions. One-way analysis demonstrated significant differences among the tissue groups for both indices ($P < 0.001$). Comparison of the indices between the tissue group using Mann–Whitney tests demonstrated that both AIs and PIs were significantly higher in hyperplasia than in apparently normal epithelium ($P = 0.044$ and $P = 0.0005$, respectively), in atypical hyperplasia than in hyperplasia ($P = 0.010$ and $P = 0.048$, respectively), and in invasive carcinoma than in *in situ* carcinoma ($P < 0.001$ and $P < 0.001$, respectively). There was no statistically significant differences between atypical hyperplasias and *in situ* carcinomas for either AIs or PIs ($P = 0.065$ and $P = 0.057$ respectively). Representative box plots for AIs and PIs in breast tissue are shown in Fig. 3.

Proliferative/apoptotic index

The ratio of PI to AI was calculated for each individual case. Cases that did not have apoptosis were excluded from the calculations of P/A index. The A/P indexes for each tissue group are summarized in Table 2. Kruskal–Wallis analysis of variance showed significant differences among the tissue groups for both indices ($P < 0.001$). Comparison of the P/A indexes between tissue groups by Mann–Whitney tests showed that P/A index increased significantly from apparently normal epithelium to hyperplasia, atypical hyperplasia, *in situ* carcinoma and invasive carcinoma ($P = 0.016$, $P = 0.043$, $P = 0.005$, $P = 0.0007$, respectively), and decreased (not significant) from hyperplasia to atypical hyperplasia and from hyperplasia to *in situ* carcinoma ($P = 0.08$ and $P = 0.064$, respectively). The P/A index increased significantly ($P = 0.046$) from *in situ* carcinoma to invasive carcinoma. When atypi-

Figure 2



Ki-67 immunostaining in ductal invasive breast carcinomas. **(a)** Positive neoplastic cells and **(b)** negative neoplastic cells, satisfying morphological criteria of apoptosis. **(c)** Positive and negative apoptotic body in the same field satisfying morphological criteria of apoptosis Avidin-Biotin-Peroxidase complex; 1000× each.

Table 1

The AIs and PIs per histological breast lesion

Tissue type	n	AI (%)	PI (%)
Normal epithelium	32	0.00 (0.00–0.40)	0.10 (0.0–3.0)
Hyperplasia	43	0.02 (0.00–0.50)	1.22 (0.0–5.5)
Atypical hyperplasia	17	0.16 (0.08–0.84)	2.02 (1.8–9.3)
Carcinoma <i>in situ</i>	25	0.52 (0.13–2.06)	6.65 (1.3–24.0)
Carcinoma invasive	50	1.17 (0.16–3.69)	17.00 (1.0–50.0)
Total	167	0.21 (0.00–3.69)	2.26 (0.0–50.0)

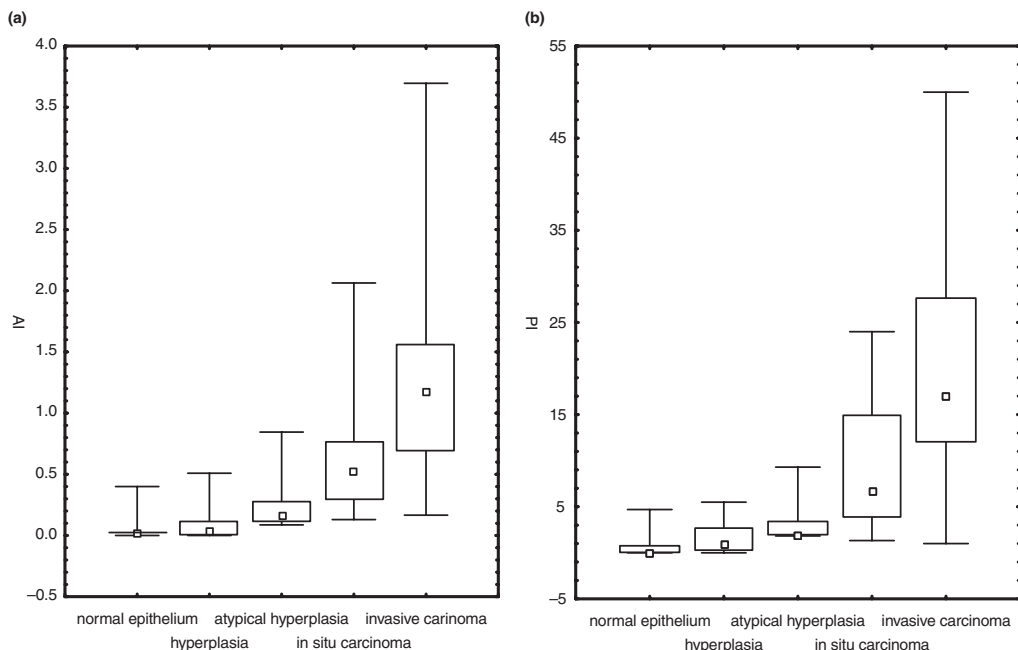
Values are expressed as median (range).

cal hyperplasia and *in situ* carcinoma were included in one group (preinvasive lesions), the P/A index decreased (not significant) from hyperplasia to preinvasive lesions ($P = 0.44$) and increased significantly from preinvasive lesions to invasive carcinoma ($P = 0.041$; Fig. 4).

Correlation between apoptotic and proliferative indices

A strong positive correlation between AI and PI was found when all cases were taken into consideration by Spearman’s test ($r = 0.83$; $P < 0.001$). When each group of lesions was analyzed separately, Spearman’s correlation demonstrated that AIs and PIs remained directly correlated in the groups of typical hyperplasia ($r = 0.45$; $P = 0.041$), preinvasive lesions ($r = 0.7$; $P = 0.001$) and invasive carcinoma ($r = 0.26$; $P = 0.046$). The correlation index was not reliable in the normal epithelium group, because of many zero values.

Figure 3



Box and whisker plot depicting the median values, interquartile range and full range of (a) AIs and (b) PIs in normal epithelium, hyperplasia, atypical hyperplasia, *in situ* carcinoma and invasive carcinoma.

Table 2

The P/A index per histological breast lesion

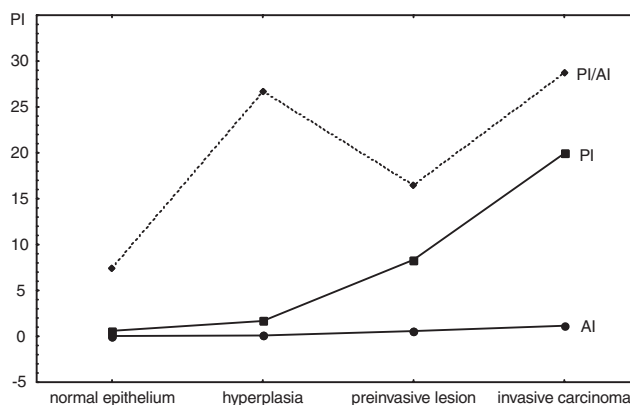
Tissue type	n	P/A index
Normal epithelium	32	1.71 (0.00–46.00)
Hyperplasia	43	20.72 (0.74–48.67)
Preinvasive lesions	42	14.11 (3.67–58.00)
Carcinoma invasive	50	20.86 (3.87–301.00)
Total	167	16.66 (0.00–301.00)

Values are expressed as median (range).

Discussion

Accumulating evidence suggests that breast cancer arises through a series of epithelial changes that range from hyperplasia with and without atypia, followed by *in situ* carcinoma to invasive carcinoma [6–9], although *de novo* breast carcinogenesis cannot be ruled out. The *in vivo* role of cell kinetics in the pathogenesis of breast cancer is still unclear. To the best of our knowledge the present study is the first to investigate apoptotic activity, proliferative activity and the balance between them in the spectrum of ductal epithelial lesions in breasts in which preinvasive lesions and/or invasive carcinomas developed. More than one lesion per case were evaluated. Each lesion was studied in a different tissue area to avoid the

Figure 4



Interaction plot of AI, PI and P/A index in normal epithelium, hyperplasia, preinvasive lesion and invasive carcinoma.

paracrine effect because both apoptosis and cell proliferation are active processes influenced by a wide variety of intracellular and extracellular regulatory stimuli [1,2].

We found that an increase in the apoptotic and proliferative activity occurs in parallel with progression of epithelial lesions. This provides further evidence for the continuum in the pathogenetic process that leads to invasive ductal

carcinoma. A quantitative study of apoptosis and cell proliferation on a static-cell population, such as tissue sections, provides little information on the actual rapidity of cell replication and loss. However, the present findings of increasing frequency of apoptosis and cell proliferation suggest the existence of an accelerating cell turnover along the multistep process of breast carcinogenesis, which could facilitate genetic events and clonal expansion. Although the cause of breast cancer is unclear, mammary tumorigenesis is believed to proceed through accumulation of specific genetic alterations.

The PIs found in the present study are comparable to those obtained by previous reports using similar methods [20,21]. The present results on the incidence of apoptosis using *in situ* visualization are generally in agreement with a previous study reported by Mustonen *et al* [22], who evaluated this cellular event in the complete spectrum of epithelial lesions using the same method. In our series, which consisted of more hyperplastic and preinvasive lesions, the results showed significant differences among apparently normal epithelium, hyperplasia, preinvasive lesions and invasive carcinoma. Expression of the antiapoptotic bcl-2 protein exists in normal ductal epithelium, and decreases from normal epithelium to *in situ* carcinoma and from *in situ* to invasive carcinoma [23]. The expression of this protein is also related to the degree of differentiation of neoplastic breast epithelium [13,14]. Interestingly, apoptotic activity was found to increase with disease progression, from dysplasia to invasive carcinoma in bronchial epithelium [24], and from normal mucosa through dysplasia to invasive carcinoma in oral mucosa [25].

In this study we also compared the rate of proliferation with that of apoptosis, because the balance between these two events and the consequences of an imbalance are fundamental to our understanding of how hyperplasia and neoplasia develop. Both diseases are characterized by a distinct increase in the number of epithelial cells. The incidence of both cellular events increases in parallel with the progression of epithelial lesions. However, an interesting point resulting from this study is that, in the transition from normal epithelium to hyperplasia and from preinvasive lesions to invasive carcinoma, proliferative activity increases more than does apoptotic activity, resulting in a growth imbalance in favour of cell proliferation.

Two studies by Harn and coworkers [16,26] reported a higher degree of apoptosis and lower proliferative activity in intraductal than in invasive carcinomas. Those investigators suggested that the higher degree of apoptosis and lower proliferative activity in *in situ* than in invasive carcinomas could result in a steady-state condition, in which net growth of the tumour is rare. The discrepancy between their results and ours, together with those of Mustonen *et al* [22], is mainly due to the higher degree of apoptosis

found in *in situ* than in invasive carcinomas by Harn and coworkers [16,26]. They used the same method, and necrotic areas were excluded from counting, as in the present study, because DNA fragmentation detected using the TUNEL method is a nonspecific marker of apoptosis. Degenerative nuclei, which may represent the final stage of cell death, either programmed or accidental, are labelled using the TUNEL method [27]. Bodis *et al* [28] and Gandhi *et al* [29] reported that, in *in situ* ductal carcinomas, the apoptotic activity correlated with lesions of high histological grade. Clinical follow-up studies [30,31] showed that, in *in situ* ductal carcinomas, the high cytonuclear grade is linked to a higher recurrence rate, and to a higher rate of progression to invasive breast carcinomas. In the present study, approximately half of the *in situ* carcinoma cases progressed to invasive carcinomas, but the number of cases was too small for a reliable statistical analysis.

The proliferative and apoptotic activities were similar in atypical hyperplasia and *in situ* carcinoma, and the ratio of proliferation to apoptosis decreased (non-significantly) from hyperplasia to atypical hyperplasia or to *in situ* carcinoma, indicating that the two lesions are kinetically similar. Molecular genetic investigations demonstrated that atypical ductal hyperplasia is a monoclonal rather than a polyclonal epithelial proliferative lesion, and it has been proposed that this lesion should be included within the spectrum of *in situ* carcinoma [32,33]. The growth imbalance in favour of apoptosis found in preinvasive lesions is difficult to explain. Early genetic events are already present in preinvasive breast lesions [32,33], and apoptosis and growth arrest are responses to DNA damage, although which of these events will occur in each instance will depend on the extent of DNA damage, cell type, location and environment. Apoptosis may be the prudent option in damaged cells that retain substantial replicative potential [4].

Furthermore, the present study demonstrated a strong positive correlation between apoptotic and proliferative activities. This finding is in accordance with previous observations on the relationship between these two activities. Studies in *in situ* and invasive breast carcinomas [34–36] showed positive correlation between apoptosis and mitotic rate, S-phase fraction and Ki-67 immunoreactivity. Similarly to invasive breast carcinomas, lymphomas, ovarian carcinomas, and hepatocellular and laryngeal carcinomas showed a strong positive correlation between these two cellular events [37–40]. Although apoptosis and cell proliferation appear to be opposing and contradictory processes, there is a recent substantial evidence [4] that the two events may be related or coupled. Early studies indicated that, under certain conditions, the process of apoptosis is associated with abnormal expression of cell cycle regulatory proteins and activation of cyclin-dependent kinases [41–43], suggesting that apop-

osis represents an abortive attempt by cells to pass through the cell cycle.

In *in situ* and in invasive breast carcinomas, in which Als and PIs were higher, Ki-67-positive cells that satisfied morphological criteria of apoptosis were observed in the present study. Because the Ki-67 antigen is expressed throughout the cell cycle [44], this observation indicates that there were apoptotic cells among the proliferative cells. There were also Ki-67-negative cells and apoptotic bodies, even in the same case. We consider that a degradation of the protein during the process of apoptosis or a nonspecific uptake of the antibody by apoptotic cells is unlikely. Therefore, these data indicate that apoptosis does not always involve entry of cells into the cell cycle. Carcinomatous breast cells can also undergo *in vivo* apoptosis when they are in the resting G₀ phase of the cell cycle. A previous *in vivo* and *in vitro* study [45] showed apoptotic bodies in lymphoid germinal centres and in intestinal crypts to be often positive for Ki-67 antigen, whereas in polymorphonuclear leucocytes and in premenstrual endometrium they were Ki-67 negative.

In conclusion, the results of this *in vivo* study indicate the following: there is increasing cell turnover along the continuum from apparently normal epithelium through hyperplasia, to preinvasive lesions and invasive carcinoma; atypical hyperplasia and *in situ* carcinoma may be kinetically similar conditions; the net increase of cells in the transition from normal epithelium to hyperplasia and from preinvasive lesions to invasive carcinoma result from growth imbalance in favour of cell proliferation; and in the transition from hyperplasia to *in situ* carcinoma there is a growth imbalance in favour of apoptosis.

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