

# Apoptotic cell death and the proliferative capacity of human breast cancers

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**Abstract.** The proliferative capacity (%S-phase fraction), DNA ploidy, apoptosis frequency (DNA fragmentation) and steroid hormone receptor status (estrogen receptor, ER; progesterone receptor, PR) of 110 samples of human breast tissues with ductal invasive carcinoma were measured using biochemical and cytofluorimetric procedures. The DNA fragmentation had a left-skewed frequency distribution and an overall median value of 1.64%, whilst the median %S-phase fraction was 8%. The median %DNA fragmentation and %S-phase fraction were 1.96% and 16% in hyperdiploid tumours ( $n = 29$ ; DNA index  $>1.1$ ) higher than in hypodiploid tumours ( $n = 10$ ; DNA index  $<0.96$ ), 0.38% and 7.5%. DNA diploid tumours ( $n = 71$ ) had median %DNA fragmentation and %S-phase values of 1.68% and 6%, consistently lower than the median values of DNA hyperdiploid tumours. The ER content of hypodiploid tumours was about one half (median: 5.9 fmol/mg) the median values in hyperdiploid (10.6 fmol/mg) and diploid tumours (14.6 fmol/mg). This may correlate with the lowest frequency of apoptosis in hypodiploid tumours, at least when measured by biochemical methods which only detect cells in the late phases of apoptosis. In contrast, the median PR was lowest in hyperdiploid tumours than in hypo and/or diploid tumours. The %S-phase/%fragmented DNA ratio for the hypodiploid tumours was 19.7, significantly higher than the ratios for hyperdiploid (8.2) and diploid tumours (3.6). These findings indicated that there is an imbalance between proliferative capacity and cell death or growth arrest in human breast tumours. This imbalance may well be linked to a loss of steroid hormone control.

Keywords: Apoptosis, human breast cancer, DNA ploidy, DNA fragmentation, steroid hormone receptors, %S-phase-proliferative capacity

## 1. Introduction

Apoptosis is a physiological form of cell death that occurs in all tissues and in which cells committed to die undergo a series of characteristic morphological and biochemical changes [8,25,32,40]. Most of the events of the active or late phases of apoptosis are readily identified in cell cultures [14, 27,48]. However, cells of the estrogen-dependent breast cancer cell line MCF-7 do not undergo internucleosomal DNA fragmentation [35,44], which is usually considered to be a specific feature of apoptosis [33]. It is more difficult to recognize and quantify apoptosis in solid tissues under physiological or pathological conditions for several reasons. First, apoptosis itself is relatively rapid

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in tissues, compared to the latent period [6,28]. Second, apoptotic cells are efficiently removed from a tissue by phagocytic elements and/or other committed cell systems [10]. Third, most of the methods currently available do not adequately identify the early phases of apoptosis, some of which probably involve specific biochemical and morphological changes in the plasma membrane, while the cells continue to appear overtly normal and healthy. It has been shown recently that the loss of lipid asymmetry by the exposure of phosphatidylserine on the outer membrane leaflet is one of the surface membrane events of apoptosis in some cell tissues [15]. This triggers signalling and transport membrane-bound enzymes in lymphoid cells [19] and activates the sphingomyelin cycle [20] with release of ceramide [21]. The better known changes, such as DNA fragmentation, chromatin condensation, membrane permeability changes and impaired mitochondrial activity [37,45] occur later. Cancer tissues undergo additional changes, such as accelerated removal of apoptotic cells, mutation of tumour suppressor genes, abnormal expression of the death inhibiting gene bcl-2 [5,38], loss of Fas expression and function [24], and inactivation of ICE and other cytoplasmic proteases [9]. All these may greatly reduce apoptosis in neoplastic tissues. They may also explain why there appears to be so few apoptotic cells in many tumour tissues [43,46]. It has also been reported that the measurable rate of apoptosis is lower in human cancer tissues than in resting or physiologically active tissues, e.g., in pregnancy, or *in vitro* tumour cell systems [5,6,16,17,19,27,30]. Other biologic properties, such as the proliferation rate and the growth fraction [12,23], the invasive potential [13], the receptor status together with endocrine and paracrine signals [29] and altered interactions with the surrounding extracellular matrix [31,34] may influence the capacity of tumours to escape from control mechanisms and avoid apoptotic death. But there have been few reports on the frequency of the apoptotic process in human breast cancer. We have therefore attempted to quantify cell death and the proliferative capacity, DNA ploidy and receptor status to provide a clearer biological profile of invasive mammary carcinoma, when a patient first presents with this disease.

## 2. Material and methods

A total of 110 samples of invasive carcinoma were obtained from breast biopsies or from mastectomy of untreated patients (age range: 30–65 years) performed at the Institute of Pathology. The histologic diagnosis was invasive ductal carcinoma in most of the cases analyzed (92%) and lobular carcinoma for the remaining 8%.

### 2.1. Tissue fractionation

Each tumour tissue sample was cut into small pieces, frozen in liquid nitrogen and ground to a powder in liquid nitrogen. The pulverized tissue sample was divided into two aliquots, a large one for measuring estrogen receptors (ER), progesterone receptors (PR) and DNA fragmentation, and a small fraction (80–100 mg) for determining the %S-phase and DNA ploidy. The pulverized tissue samples were suspended in Tris/K/Mg buffer and homogenized with a teflon Potter homogenizer (3 times up and down at 1000 rpm at 4°C). The homogenate was centrifuged at  $100,000 \times g$  for 30 min at 4°C in a Beckman L5 ultracentrifuge. The supernatant was carefully recovered and assayed for ER and PR receptors by the Ligand Binding Assay (LBA), the “gold standard” biochemical method validated by the EORTC Receptor Group [4]. The microsomal pellet was incubated for 1 h at 50°C in 10 mM Tris, 10 mM Na<sub>2</sub> EDTA, 0.4% SDS, 150 mM NaCl, pH 7.4, 50 µg/ml proteinase K. 10 µl of DNase-free RNase A (50 µg/ml) were then added and incubation continued for 1 h at 37°C, plus 5 min at 70°C.

An aliquot (20  $\mu$ l) of the microsomal lysate was examined by agarose gel electrophoresis for DNA fragments and the main sample was ultracentrifuged at 15,000  $\times$  g for 30 min at 4°C. The supernatant containing fragmented DNA was separated from the intact DNA in the pellet before DNA assay.

### 2.2. Analysis of DNA fragmentation. Colorimetric method

The DNA contents of both fractions obtained by ultracentrifugation were measured by a stoichiometric diphenylamine reaction [7]. The absolute amount of DNA was determined by comparison with a standard reference curve generated from calf-thymus (linear: 2–80  $\mu$ g) and by reading the optical density at 595 nm. The percentage (%) DNA fragmentation was calculated by the ratio ( $\mu$ g supernatant DNA)/( $\mu$ g supernatant DNA +  $\mu$ g pellet DNA)  $\times$  100.

### 2.3. DNA electrophoresis

DNA was separated by electrophoresis on 1.5% agarose gels in TBE buffer containing 89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.4 at 10 V, 30 mA [19]. An aliquot of microsomal lysate (20  $\mu$ l) was mixed with 10–20  $\mu$ l solution containing 0.05% bromophenol blue, 40% sucrose, 100 mM EDTA and 0.5% SDS. Standard DNA ( $\lambda$  EcoRI/Hind III digest 125–21226 bp) was used as control. Gels were stained with 0.5  $\mu$ g/ml ethidium bromide, examined under UV light and photographed with a IBI quick shooter device.

### 2.4. Measurement of %S-phase and DNA ploidy

Aliquots (80–100 mg) of frozen tumour powder was gently dispersed in 1.5 ml 0.04 M Tris, 0.5 M sucrose, 1.5 mM MgCl<sub>2</sub> pH 7.4 using a loose-fitting teflon pestle. The resulting homogenate was filtered through several layers of nylon (mesh: 210 and 53  $\mu$ m). The filtrate was layered onto a double layer of sucrose (1.5 and 1.75 M) according to Dressler et al. [12] and centrifuged at 1500  $\times$  g for 45 min at 4°C. The supernatant was removed and the nuclei (in the pellets) were washed with minimal essential medium (MEM) containing 10% fetal calf serum (FCS). There were  $2 \times 10^4$ – $2 \times 10^6$  nuclei per 100 mg frozen pulverized tumour. The nuclear fraction was lysed and DNA stained by mixing the nuclei with 0.1% sodium citrate, 0.3% Nonidet P (NP-40), 0.05 mg/ml propidium iodide and 0.02 mg/ml RNase A (Sigma) for 10 s, and incubated for 30 min at 40°C. The fraction was centrifuged and nuclei resuspended in fresh staining solution, homogenized by passage through a 27-gauge needle and finally filtered through 37- $\mu$ m nylon mesh immediately before measuring in a Becton Dickinson (BD) FACscan flow cytometer. The %S-phase and DNA index (DI) were estimated after collection of at least 10,000 events with a BD Cell-Fit program and the polynomial model for calculations. %S-phase was measured in the first aneuploid (hypodiploid or hyperdiploid) populations. %S-phase values of additional diploid subpopulations were excluded from the group of truly diploid tumours. DNA diploid tumours had a DI of 1; DNA aneuploid tumours had a hypodiploid DNA content when the DI was less than 0.96, and a hyperdiploid DNA content when the DI was 1.1–1.9. The mean coefficient of variation (CV) at the G0/G1 mean peak was always less than 5% [41]. Isolated human peripheral blood lymphocytes fixed with ethanol were used as internal diploid DNA/G0-G1 control cells.

### 3. Results

Table 1 summarizes the values obtained for the %S-phase, %DNA fragmentation, estrogen (ER) and progesterone (PR) receptors for the 110 samples of breast tissue with invasive carcinoma, and their division into three groups according to the DNA ploidy status. All the values varied greatly, indicating a non-Gaussian distribution in all tumour groups, as illustrated in the histogram plots (Figs 1 and 2). Hence, they can be adequately described by their median values (Table 2). The median value of %S-phase fraction for the entire population was 8%, quite close to the overall median value of 5.8% reported for 1084 breast tumours [12]. The lowest median value of %DNA fragmentation (0.38%) was found for apoptosis in breast tumours with a DNA hypodiploid index, whilst the highest (2.57%) was measured in tumours with a diploid DNA index, but with lymph node metastases. DNA hyperdiploid tumours had a median %DNA fragmentation 4 times higher than that of the DNA hypodiploid group. They also had the highest median %S-phase fraction (16%) which was 1.5-fold higher than the median reported by Dressler et al. [12]. The greatest value of %DNA fragmentation (62%) was measured only once, in a DNA hyperdiploid tumour. DNA diploid tumours had the lowest median proliferative capacity (6%), regardless of the metastatic status, but lymph node involvement was associated with the highest median DNA fragmentation and the highest median ER concentration (Table 2). However, microsomal fractions prepared from these tumours and analyzed for DNA by agarose gel electrophoresis did not show evidence of ladder suggestive of internucleosomal fragmentation, nor of non-random DNA cleavage into large fragments (>22 kbp) (Fig. 3). Analysis of the relationship among parameters indicated an imbalance in favour of proliferation, especially for the DNA hypodiploid tumours, as indicated by the %S-phase/%DNA fragmentation ratio. Its value contrasted with the correspondent ER/PR ratio which was close to one, as the median values for both receptors were low in this group (Table 2). Hyperdiploid DNA tumours had the highest ER/PR ratio (4.1), but only a positive ER median value.

Table 1  
Range values for %S-phase, %DNA fragmentation, ER/PR receptors measured in DNA hypodiploid, diploid and hyperdiploid breast cancer tumours

	Hypodiploid ( <i>n</i> = 10)	Diploid ( <i>n</i> = 71)	Hyperdiploid ( <i>n</i> = 29)
%S-phase	2.0–18.0	0.4–38.0	3.0–56.0
%DNA fragmentation	0.0–29.9	0.0–16.4	0.0–62.0
ER (fmol/mg)	0.1–149	0.0–165	0.1–304
PR (fmol/mg)	0.0–39.1	0.0–483	0.0–93.6

*n* = number of cases.

Table 2  
Median values  $\pm$  1 standard error of the median for %S-phase and %DNA fragmentation

DNA	<i>n</i>	%S-phase	%DNA fragmentation	%S-phase/%DNA fragmentation	ER	PR	ER/PR
Overall diploid	71	6 $\pm$ 0.7**	1.68 $\pm$ 0.1	3.6	14.6	6.2	2.3
Diploid met –	55	6 $\pm$ 0.8**	1.38 $\pm$ 0.2	4.3	11.2	4.9	2.3
Diploid met +	16	6 $\pm$ 2.0**	2.57 $\pm$ 0.4	2.3	28.4	9.2	3.1
Hypodiploid	10	7.5 $\pm$ 4.5*	0.38 $\pm$ 0.2*	19.7	5.9	5.0	1.2
Hyperdiploid	29	16 $\pm$ 2.7	1.96 $\pm$ 0.5	8.2	10.6	2.6	4.1

ER and PR, estrogen and progesterone receptors; met, metastases.

\* $2p < 0.01$ , \*\* $2p < 0.001$  significantly different when compared to the hyperdiploid values by the Mann–Witney *U*-test.

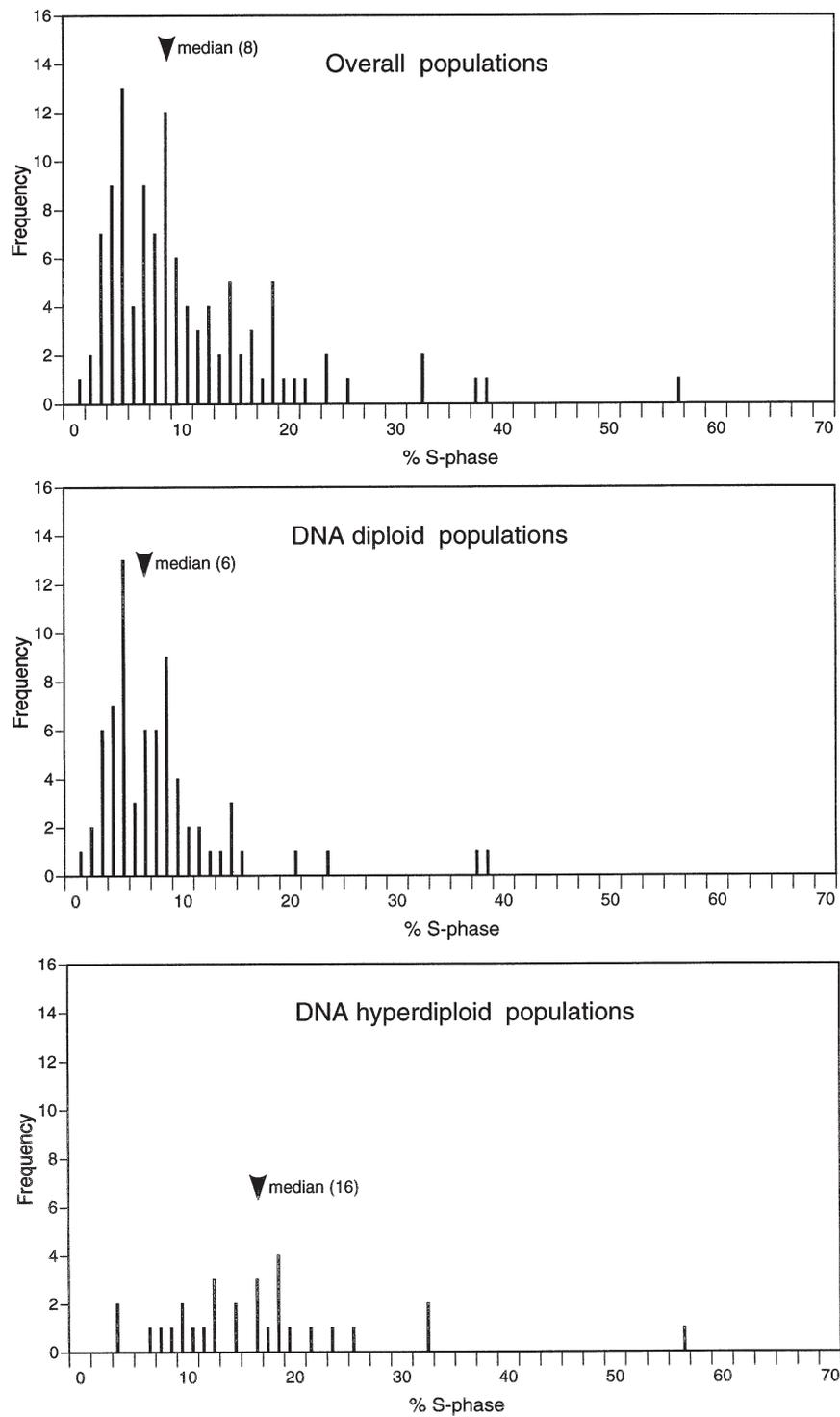


Fig. 1. Frequency distributions of %S-phase values for 110 samples of breast tumours and for the tumours divided according to the DNA ploidy.

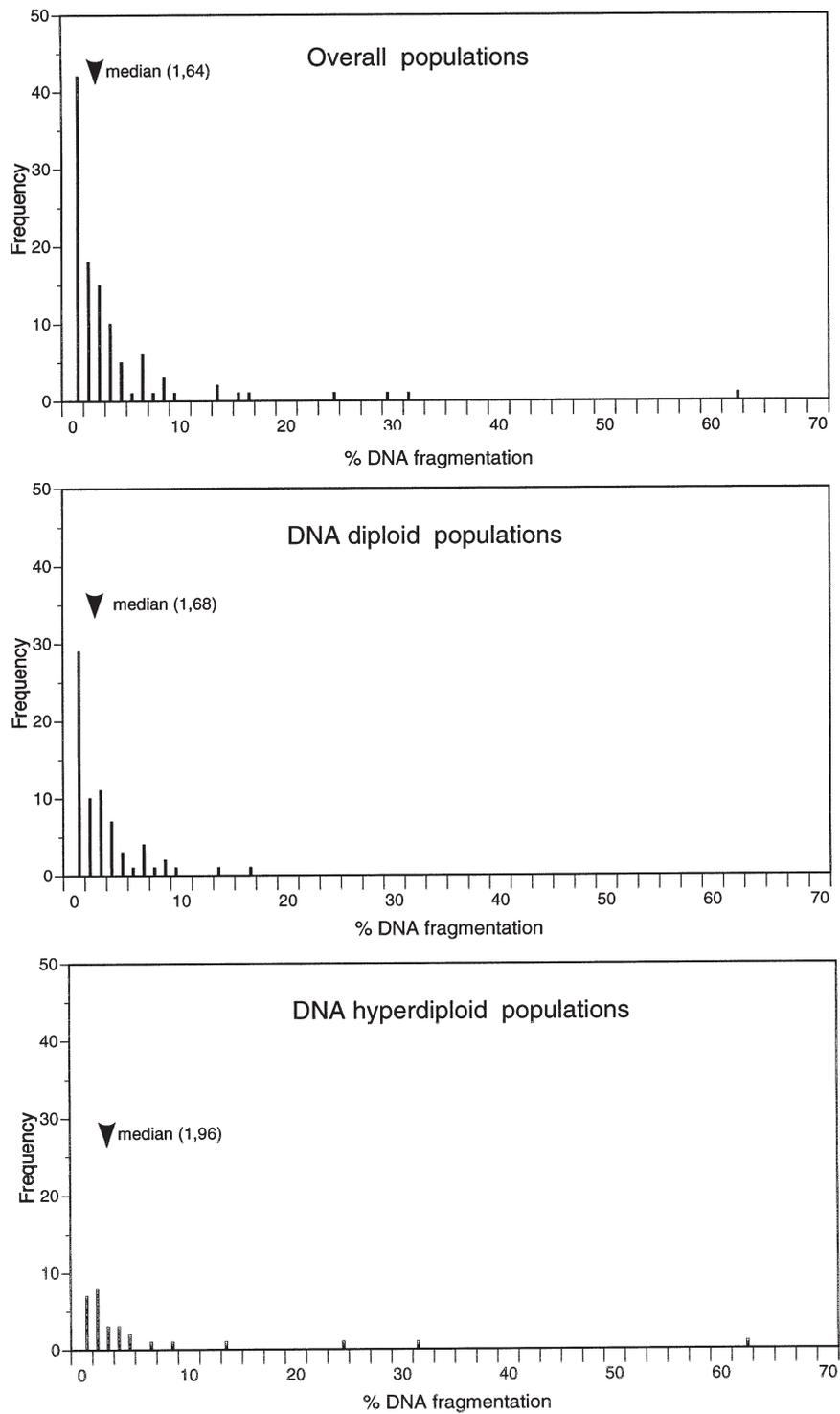


Fig. 2. Frequency distributions of %DNA fragmentation values for 110 samples of breast tumours and for the tumours divided according to the DNA content.

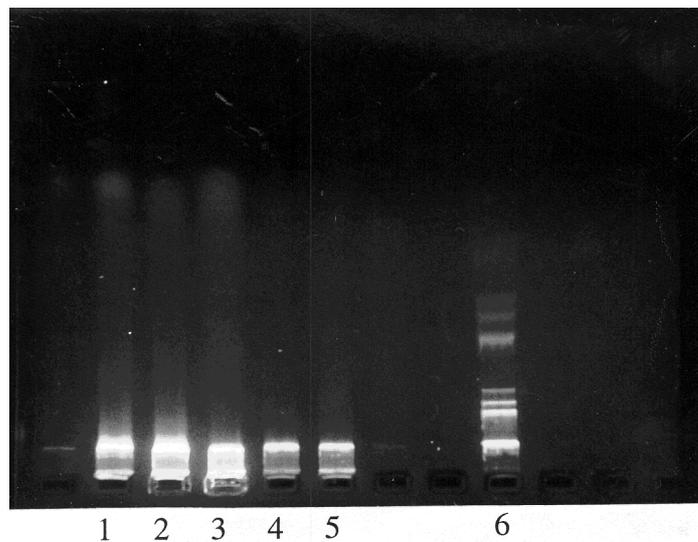


Fig. 3. Electrophoretic analysis of DNA fragments on a 1.5% agarose gel. Lane 1, Ca with DI 0.85; lane 2, Ca with DI 1.25; lane 3, Ca with DI 1.72; lane 4, Ca with DI 2.51; lane 5, Ca with DI 2.69; lane 6, standard DNA ( $\lambda$  EcoRI/Hind III digest 125 bp–22 kbp). Ca, carcinoma; DI, DNA index.

#### 4. Discussion

The results of the present study indicate that the frequency of measurable apoptosis is low in most human breast tissues with invasive carcinoma. Apoptosis was quantitatively assessed in microsomal membranes obtained by ultracentrifugation of homogenates from frozen breast tissue specimens by determining the fragmented DNA. Although percentage DNA fragmentation values varied greatly, the median frequency of apoptosis ranged from 0.38 to 2.57% for all tumours regardless of the DNA ploidy, but the lowest median frequency was in breast tumours with a hypodiploid DNA index. This residual apoptosis measured biochemically on 110 breast carcinoma specimens is consistent with recent data indicating an average apoptotic frequency of 1%, determined by morphometry on histological sections from 15 cases of invasive breast cancer [43]. A previous study aimed to determine the proliferative activity within the epithelial cells of histologically “normal” human breast and of histologically diagnosed pathology (fibroadenoma, fibrocystic changes, carcinoma) in the same breasts has shown that the apoptotic index (AI) measured on histological sections was reduced in “normal” epithelium from breasts having fibrocystic changes (0.17%) and carcinoma (0.19%) compared to “normal” breasts with fibroadenoma alone (0.27%) and to neoplastic tissues, namely, fibroadenoma (0.27%) and carcinoma (0.42%) [1]. Thus, apoptosis seems to be a relatively rare phenomenon in breast cancer and its frequency is significantly lower than in several cell culture systems. For example, most MCF-7 cells deprived of estrogen for 10 days became detached and showed non-random cleavage of nuclear DNA into the 50 and 300 kbp fragments typical of apoptosis, but very few had the classical morphology of apoptotic cell death or internucleosomal DNA fragmentation [44]. The cells remaining attached were morphologically viable and had no apoptotic features [44]. Preliminary results obtained in our laboratory indicate that most MCF-7 and estrogen-independent SKBR-3 mammary cancer cells blocked in the G0 and/or G1 phase by treatment for 4 days with the  $\text{Ca}^{++}$  ionophore A23187 (calcimycine) undergo apoptosis, with classical ultrastructural features, DNA fragmentation, altered permeability and a subdiploid region of cell cycle. The majority of thymocytes and human peripheral

blood mononuclear cells (PBMNC) cultured for three days with the glucocorticoid dexamethasone contain apoptotic figures [19,48]. Physiologically active breast tissue contains apoptotic cells but has an apoptotic frequency consistently less than the mitotic frequency [3]. The mean number of apoptotic figures per lobule in the breasts of pregnant women, assessed by morphological inspection varied from 0.41 to 1.13 throughout pregnancy, whilst the breasts of lactating women had a mean frequency of only 0.04. There was a direct correlation with the mitotic frequency, which was 1.23–1.43 in the breasts of pregnant women, and only 0.02 in the breasts of lactating women [3]. However, mammary gland involution, which follows weaning, occurs mostly by apoptosis [42]. The frequency of measurable apoptosis may be generally low in normal and neoplastic tissues because this is a natural process which is residual in living tissues. The real rate of cell death in tissues cannot be readily estimated probably because apoptosis is very rapid and most apoptotic cells are swiftly removed, thus escaping detection [6]. Other authors have previously reported that apoptosis is a rapid process in human as well as in animal tissues [16,36]. Second, the rate of removal of apoptotic elements by phagocytes may vary with the ploidy and proliferative status of the tissue. On the other hand, *in vivo* abrogation of apoptosis may concern cell elements that are committed to death, but are in the latent or initial stages of the process, and so undetectable by the methods presently available [28]. The extremely low residual apoptosis in hypodiploid DNA tumour tissues (0.38%) is inversely related to their proliferative capacity, as indicated by the %S-phase. In these tumours the median %S-phase is slightly higher (7.5%) than the median %S-phase (6%) of diploid DNA tumours, but is only half that of hyperdiploid DNA carcinomas (16%), which also have the highest percent apoptotic cells (1.96%). The optimal median cut-off point for S-phase fraction was set at 7% for DNA diploid cases and 12% for the DNA aneuploid cases, so that our results (6 and 16%, respectively) are well in line with proliferative capacity found in previous reports [12,23]. Our findings suggest that there is an imbalance between residual apoptosis and cell growth in breast tumours when compared to physiologically active and resting human breast tissues [3,16], as documented by the %S-phase/%DNA fragmentation ratio. This is particular high in hypodiploid DNA tumours. The hypodiploid and hyperdiploid DNA tumours also had low levels of ER and PR, indicating that apoptosis is no longer or poorly regulated by estrogens in these aneuploid carcinomas. It has been shown recently that the expression of the bcl-2 gene, which controls apoptosis, is mainly associated with the steroid receptor status and with low grade/estrogen-receptor positivity in slowly proliferating node-negative breast carcinomas [2] but inversely correlated with the Ki-67 growth fraction in breast carcinomas [11]. Clinical studies have shown that there is a correlation between the synthesis of anti-apoptotic bcl-2 protein and the estrogen receptor content of normal tissues [39] and invasive breast carcinomas [18,22]. When cells of the human breast cancer cell line MCF-7 are exposed to  $\beta$ -estradiol, their content of bcl-2 mRNA is increased, but not that of bax mRNA, indicating that steroid sex hormones can inhibit apoptosis by stimulating the production of the anti-apoptotic protein bcl-2 [44]. In conclusion, the routine determination of residual apoptosis in breast tissues with invasive carcinoma may not yet have any clinical relevance, because the morphologically apparent process is rare in these tumours, at least as measured by conventional procedures. However, if adequate methods become available that can identify and quantify the early or initial phases of apoptosis, similar to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane in lymphocytes [15], or the activation of signal transduction and transport membrane enzymes [19], then assessment of the apoptotic potential could become relevant [26]. It could give a clearer indication of spontaneous cell loss, the proliferative capacity, the susceptibility to chemical treatment and, finally, the aggressiveness of a breast tumour, and thus provide a predictive information.

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