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Spontaneous Apoptosis in the Intra-Ductal Component's Stroma of Breast Invasive Carcinoma

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Abstract. *Spontaneous apoptosis by in situ detection of DNA fragmentation (DNAf) was investigated in breast invasive ductal carcinoma (IDC) frozen samples removed from 61 untreated patients. The incidence of DNAf was low in carcinoma cells and was mainly detected in the stroma. In the stroma at a distance from carcinoma cells, DNAf was inversely related to estradiol plasma level variations (p=0.01), indicating that it probably remained under physiological hormonal regulation. In the stroma adjacent to carcinoma cells, DNAf was correlated to tumor progression parameters such as the presence of a comedo intra ductal carcinoma (DCIS) component (p=0.001) and axillary lymph node metastasis (p=0.002), suggesting that this stromal compartment more probably represented a tumoral component closely associated to epithelial tumor cells. Therefore, the detection of DNAf in the adjacent stroma of breast carcinoma could help to predict progression in non invasive tumors and also in invasive tumors in those patients without lymph node invasion.*

Apoptosis is a distinctive form of cell death manifested by characteristic chromatin condensation and DNA fragmentation, whose function is to eliminate unwanted host cells. In contrast to necrosis, it is considered as a normal homeostatic mechanism to maintain cell populations during development or in adult tissues (1). Apoptosis is the end-point of a cascade of molecular events, internally programmed by a dedicated set of genes products. In hormonally-regulated tissue like the breast, epithelial cell loss at the end of the menstrual cycle or during involution of lactating breast takes place through apoptosis (1). An inverse link between sex steroid hormones and apoptosis is supported as the antiapoptotic bcl-2 protein varies with the menstrual cycle (2) and expression of the estrogen receptor (ER) has been correlated with bcl-2 protein expression (3). *In vitro* investigations on breast cancer cell lines have demonstrated down-regulation of apoptosis by estrogen, supporting that estrogen-induced breast cancer cell growth may be due not only to an agonist effect on cell proliferation but also to deregulation of programmed cell death (4).

Several studies of human breast tumors have reported the extent and prognostic value of this process. Apoptosis has been found in spontaneous breast carcinoma with active cell growth indicated by high histological grade, high proliferation rates (5) or aneuploidy (3). Since it was not predictive of a worse prognosis (5), it is likely that apoptosis counterbalances cell proliferation and probably delays tumor growth (1).

By contrast, little data exists concerning apoptosis in the tumor stroma which is actually believed to play a key role in tumor progression. Studies performed over the last few years in the breast have reported that stromal cells, in the neighbourhood of cancer cells, may produce various proteinases that facilitate tumor invasion through their ability to degrade extracellular matrix (6). These findings, together with the observation that mammary gland involution by

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Table I. *Clinicopathological data on breast IDC.*

Characteristic	n
Patient age	61
≤ 40 years	5
> 40 years	56
Pathological tumor size	42
≤ 10 mm	3
11-20 mm	21
> 20 mm	18
Menopausal status	61
Pre	24
Post	37
Histological grade of IDC	56
Ductal grade I	13
Ductal grade II	28
Ductal grade III	15
Holland grade of DCIS	29
Low	6
Intermediate	5
High	18
Pathological nodal involvement	56
Negative	37
Positive	19
Estrogen receptor status	61
≤ 10 fmol/mg	17
> 10 fmol/mg	44
Progesterone receptor status	61
≤ 10 fmol/mg	19
> 10 fmol/mg	42

apoptosis is also associated with extracellular matrix remodeling (7), suggest a link between apoptosis in the tumor stroma and breast cancer progression.

In this study, we evaluated DNA fragmentation *in situ* in breast carcinoma samples in order to extend previous knowledge concerning the occurrence of cell death in epithelial tumor cells but also in the stroma, and to explore correlations with clinicopathological prognostic parameters.

Materials and Methods

Tissues and patient characteristics. Breast frozen tumor specimens were randomly chosen from within a large pool of invasive ductal carcinoma (IDC) of the usual type from 61 patients without any radiation or chemotherapy prior to surgery. Clinicopathological data on these tumors are reported in Table I. Tumor samples were obtained from the Cancer Research Centre (Centre Val d'Aurelle) of Montpellier (France). Specimens were snap-frozen in liquid nitrogen upon arrival in the pathologist's laboratory, *i.e.* within 15 minutes after surgical removal, and stored at -70˚C until use. All tissues were collected for therapeutic or diagnostic purposes according to the ethical rules of Helsinki (1984), modified in Tokyo, and with the approval of the local ethics committee. Breast IDC in the World Health Organisation Histological (WHO) Typing of Breast Tumors were graded according to the Bloom-Richardson method (8) and axillary lymph node involvement was determined by histological examination. An intra ductal carcinoma (DCIS) component was present in 45 IDC. Concentrations of α subunit of estrogen receptor (ERα) and progesterone receptor (PgR) were determined as described (9). Plasma estradiol (E2) and progesterone levels were performed as described (2).

In situ terminal deoxynucleotidyl transferase (TdT) assay. DNA fragmentation (DNAf) in tissue was detected using an isotopic *in situ* procedure (10). For every frozen tumor sample, DNAf assay was performed 3 times in independent experiments. In each experiment the 38B9 cell line (11), in which apoptosis was induced by treatment with Calcium Ionophore (10 mM, Cabiochem), was used as a positive control. The 5-μm cryosections from 61 breast samples and 38B9 cell pellet were mounted on poly-L-lysine-coated slides. After fixation in 4% paraformaldehyde, sections were dehydrated in a graded series of alcohol and stored at -70˚C. When used for DNA fragmentation assay, slides were prewarmed at 37˚C after 2 minutes rehydratation in PBS (1M). Each section was covered with 200 μl of a mixture containing 10 mCi of ^{33}P dATP (3000Ci/mmole, Amersham, France), 1 x reaction buffer pH6 (5 x reaction buffer is : 1M potassium cacodylate, 0.125 M Tris-HCl and 1.25 mg/ml BSA), 2.5 mM cobalt chloride, and five units of terminal transferase (Boerhinger Mannheim, Germany). After 45 minutes of incubation at 37˚C in a moisture chamber, slides were dipped for 15 minutes in PBS (1M) at room temperature, 10 minutes in 0.1M SSC at 65˚C and finally in 0.1M SSC at room temperature. After dehydratation in a graded series of alcohol, slides were dipped in Ilford K5 emulsion for autoradiography previously melted at 40˚C. They were drained and exposed at 4˚C for 14 days. After exposure, the slides were developed for 2 minutes in Kodak D19 developer (USA) at 22˚C, rinsed in 2% acetic acid and fixed for 5 minutes in Kodak L4 fixer. Finally, the tissue sections were counterstained with haematoxylin. For assessment, sections were examined under conventional conditions by two authors (C. Rouleau and C. Escot). Apoptotic cells were identified in each section in carcinoma cells, in the stroma adjacent to tumor cells (stroma located up to 550 μm from carcinoma cells) and in the stroma at distance from tumor cells (stroma located between 550 μm to 1.5 mm from carcinoma cells). In epithelial tumor cells, results were expressed as a percentage of labeled cells in at least 100 cells in high power fields. Necrotic nuclear karyorrhexis and apoptotic cells in the necrotic foci or glandular lumina were not counted, even when they showed positive signal, because of their unknown origin. In the adjacent and in the distant stroma, unequivocal nuclear labeling was regarded as representing an apoptotic cell when observed in a non inflamed background (-, +/-, + :refers to no detectable, weak and high nuclear signal).

Statistical analysis. Data were stored using the Paradox 4,5 database management system (Borland, Scotts Valley, CA, USA) on an IBM compatible PC. Statistical analysis was performed with ANGOSS KNOWLEDGE SEEKER software (Angoss Software Intl Ltd, Toronto, Canada). Statistical differences were determined using F-tests for ANOVA (2) and Chi-square calculation. Fisher's exact test was substitute for Chi-square when an expected value was lower than 5.

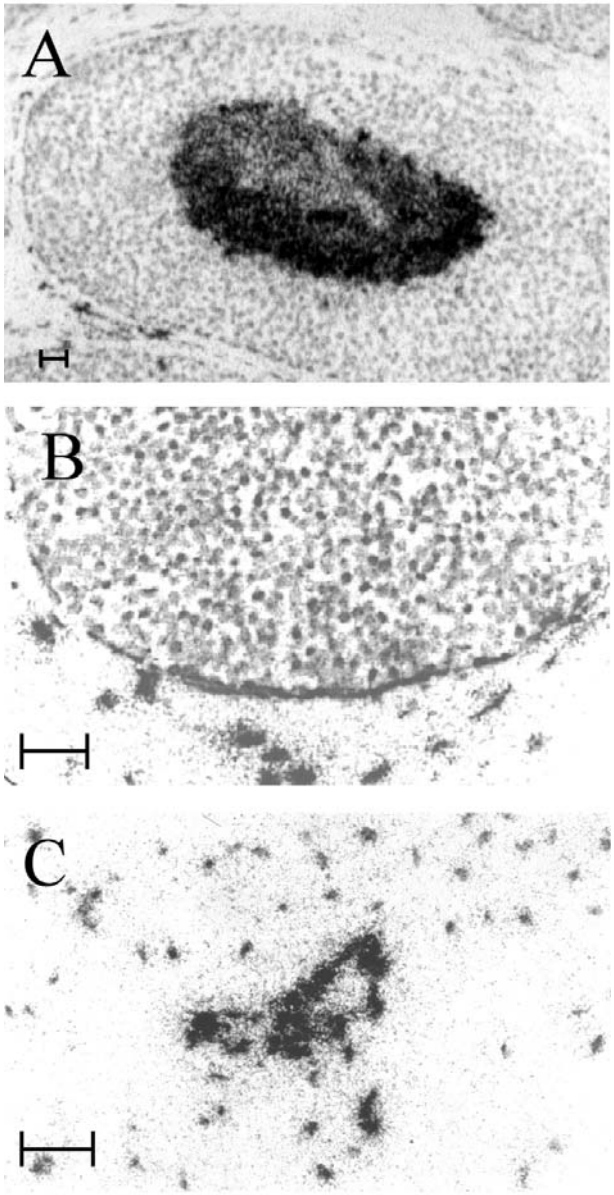

Figure 1. *Localisation of the DNA fragmentation (DNAf) signal in breast ductal carcinoma samples. DNAf in epithelial tumor cells and in the adjacent stroma of a comedo intra ductal carcinoma DCIS (A), in the adjacent stroma of no comedo DCIS (B) and in the distant stroma (C). Scale bar=50 μm.*

Results

Assessment of the 61 IDC samples after light haematoxylin staining identified 101 histological structures consisting of 51 IDC and 50 DCIS. Among the DCIS structures, 22 showed central necrosis reminiscent of comedocarcinoma.

Eight out of the 61 (13.1%) cases studied displayed foci of morphologically viable carcinoma cells positively stained by DNAf assay. A DNAf signal in more than 1% of carcinoma cells was seen in 3 out of 51 (5.9%) IDC. Scattered epithelial tumor cells with clear nuclear labeling were detected in 8 out of 50 (16%) DCIS. In 6 out of 22 (27.2%) intra ductal comedocarcinomas, a DNAf signal was associated with isolated epithelial tumor cells at the periphery of the duct, in the viable rim of carcinomatous epithelium. Necrotic areas commonly found inside the ducts showed strong diffuse non-specific labeling, without sharp demarcation between cell borders (Figure 1A). DNAf in carcinoma cell was not correlated with any clinicopathological data.

Table II. *DNA fragmentation signal in the adjacent stroma of invasive ductal carcinoma (IDC) samples.*

Parameters	N total	DNAf + n (%)	DNAf - n (%)	*P* value
Lymph node invasion				
-	37	7 (18.9)	30 (81.1)	
+	19	13 (68.4)	6 (31.6)	0.002
Intra ductal component				
-	16	0 (0)	16 (100)	0.001
+, without comedo	21	6(28.6)	15(71.4)	0.001
+, with comedo	24	13(54.2)	11(45.8)	0.001

N: no of tumor samples; DNAf: DNA fragmentation; DCIS: intra ductal.

The DNAf signal was mainly located in spindle-shaped/fusiform cells in the adjacent stroma in 22 out of 61 (36.1%) tumor samples. It was observed in the adjacent stroma to 28 out of 50 (56%) DCIS structures, with positive fibroblast-like cells surrounding epithelial tumor cells (Figure 1B). In 13 out of 51 (25.5%) tumors, signal was also detected on adjacent stromal cells located in the border between IDC and DCIS. Conversely, no labeling was observed in the adjacent stroma of IDC. Statistical analysis, presented in Table II, showed that DNAf signal in the adjacent stroma of IDC samples was significantly associated with positive nodal status ($p=0.002$) and also correlated to the presence of a DCIS component ($p=0.002$), especially of comedo type ($p=0.001$). No trend was found with other parameters such as tumor size, SBR grade or hormone receptor status.

DNAf signal was also identified in the distant stroma (Figure 1C). End labeling of spared stromal cells in 19 out of 61 (31.1%) cases was not associated with any pathological tumor parameter but was exclusively inversely correlated to plasma estradiol levels ($p=0.01$).

Discussion

We evaluated DNA fragmentation in 61 primary breast IDC in serial tumor sections. This study highlighted the low incidence of spontaneous DNAf signal in breast carcinoma

cells, in agreement with the hypothesis that malignant tumors and especially carcinoma are associated with inhibited cell death and increased cell survival (12). However, previous studies have reported a high apoptotic index in breast carcinoma cells (5, 12, 13). There are several possible reasons for these discrepancies. Evaluating the apoptotic index remains a subjective exercise like the mitotic count (12). Moreover, fixing tissues at room temperature before paraffin embedding is a time consuming process which could induce spontaneous DNAf signal in epithelial tumor cells. As the onset of apoptosis proceeds rapidly, it may be related to the degradation of unstable anti-apoptotic proteins such as bcl-2. Previous work in our laboratory showed that using frozen tissue avoids degradation of proteins involved in the cell death program such as bcl-2 (2). In the current study, only 4 high-grade DCIS out of 18 showed unequivocal DNAf signal on viable epithelial tumor cells at the periphery of the duct. In contrast to the work of Bodis *et al.* (14), who reported a strong DNAf staining in high-grade comedo DCIS and suggested that extensive necrosis is likely to represent apoptosis, we have not taken into account DNAf in central necrosis and in the adjacent surrounding band of darkly stained degenerating cells. However, *in situ* molecular procedures of cell death detection are not absolutely specific for programmed cell death. They rely on the presence of fragmented DNA in apoptotic cells and labeling of DNA strand breaks may also occur in necrosis phenomena (1). Morphological assessment remains essential to distinguish both cell death types. In comedocarcinoma, morphological features indicate that ischemia possibly occurs at the center of the duct, where neoplastic epithelium is not vascularised. In the same way, dystrophic calcifications frequently developed in necrotic areas of comedocarcinoma are not a typical feature of apoptotic bodies (1). Nevertheless, DNAf was mostly observed in the adjacent stroma of epithelial tumor cells. This is the first study, to our knowledge, reporting DNAf in the tumor stroma rather than in carcinoma cells. In all breast carcinoma studied to date, fragmented DNA is limited to epithelial tumor cells and it has never been observed in the stromal cells surrounding the neoplastic cells. However, we detected DNAf using an isotopic procedure that we found to be more sensitive as compared to non isotopic procedure (data not shown). In the distant stroma, DNAf signal was not related to any tumor parameter but was correlated to plasma estradiol levels ($p=0.01$). This suggests that cell loss in the distant stroma occurs under the control of steroid hormones, and is consistent with tissue remodeling that normal mammary gland physiologically undergoes according to the menstrual cycle (1, 2). Conversely, no trend was observed between the occurrence of DNAf in the adjacent stroma and plasma estradiol levels, indicating that this part of the stroma has lost the hormonal influence. These results are in agreement with recent considerations on the tumor stroma (15, 16). Wolf *et al.* postulated that the stroma surrounding breast epithelial tumor cells more likely represents a tumoral component since it morphologically and biochemically differs from the normal connective tissue.

Although unexpected, DNAf is more frequently observed in the adjacent stroma of DCIS, rather than in the adjacent stroma of IDC. Furthermore, it is correlated to the presence of a comedocarcinoma that has a higher risk of becoming invasive than other forms of DCIS (17). Because axillary lymph node metastasis in patients with comedocarcinoma has been reported in the absence of obvious invasion, some authors have postulated that this form of DCIS may already be invasive in a "pushing" fashion.

We therefore suggested that DNAf possibly represents pre (or early) invasive changes in the adjacent stroma of DCIS, that may be lost with progression of DCIS to invasion. One explanation is that *in situ* epithelial cells may secrete diffusible substances to degrade the basement membrane and to drive apoptosis in the adjacent stroma (18). Therefore, evaluation of DNAf may have practical implications in the management of breast tumors. In non invasive lesions, identification of the DNAf pattern in the adjacent stroma of DCIS could contribute to define sub populations of aggressive tumors. In invasive tumors, since it is correlated to axillary lymph node involvement ($p=0.002$), detection of DNAf could help to predict the outcome, especially in those patients without lymph node invasion.

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