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Frequency Analysis of Apoptosis in Sporadic Breast Cancer

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The development of breast cancer is associated with an accumulation of specific genetic alterations. These genetic changes affect malignant transformation of both dysregulation of cell proliferation and apoptosis. Apoptotic cell death is a frequent phenomenon in breast cancer. In present work we investigated the association between apoptosis and breast cancer progression.

The apoptosis was analysed in breast cancer patients (n=103) in blood and tumour specimens. Blood samples from age matched healthy women served as control (n=90). The apoptosis was detected by special staining techniques TUNEL and by agarose gel electrophoresis.

The apoptotic cells were identified in 69.9% (72/103) of the breast cancers and in 1.1% control (1/90). The number of positive samples were significantly higher among cancer samples than among control samples ($P < 0.001$). There were the significant difference in terminal deoxynucleotide transferase-mediated deoxyuridine triphisphate-biotin nick end-labelling index between ductal breast carcinoma and the other histological types. Sex steroid recepor negative tumors have greater apoptotic index than the sex steroid receptor positive ones.

The high frequency of apoptotic cells in breast tumours suggests a potential role of apoptosis in cancer appearance and/or progression.

Introduction

Breast cancer is one of the major killers worldwide. The genetics of mammary carcinogenesis has not been fully elucidated. Many factors appear to influence the growth and proliferation of breast cancer cells. Steroid hormones (oestrogens, gestagens and androgens), growth factors (epidermal growth factor, transforming growth factors α and β, fibroblast growth factor, and others) and their receptors, and various cytokines and lymphokines affect the behaviour and phenotypic expression of breast cells [7, 9]. Analysis of apoptosis in breast cancer has gained widespread acceptance as a useful prognostic approach in the management of this disease. The role of apoptosis in oncogenesis is currently being studied intensively in breast cancer.

Apoptosis and necrosis are two basic types of cell death [2]. Apoptosis, or programmed cell death, is a physiological form of cell death that plays a critical role in cell-deletion in normal homeostasis and embryogenesis [16] and contributes to retardation of tumour growth [10]. The pathogenesis of apoptosis involves the cleavage of nuclear chromatin between the nucleosomes by specific endonucleases, producing chromatin fragments composed of approximately 200 base pairs [2, 19]. Apoptosis is regulated by members of the Bcl–2 protein family, some of which such as Bcl–2, Bcl-X_L, Mcl–1 and A1, suppress apoptosis, whereas others, including Bax, Bak, Bcl-X_s, Bad, Bik, Bid, Bok, Hrk, Mtd and Boo, promote it [15, 18].

In humans Bcl–2 is expressed in about 80% of breast cancers and is correlated with the expression of oestrogen and progesterone receptors – good prognostic features in breast cancer [11]. Major correlation between Bax protein and outcome have not been observed, although studies have shown reductions in Bax to be associated with a poor response to chemotherapy in metastatic breast cancer [11, 15].

Apoptosis is increased in ductal carcinoma *in situ* and invasive breast cancer [5, 12]. Apoptosis seems to be reduced relative to proliferation in normal breast epithelium around invasive breast cancer. High level of apoptosis in tumours have been correlated with worse survival and have been reported by others to be an independent variable when all other prognostic indicators are considered [1, 4, 6, 21].

In this study, we analysed the potential role of apoptosis in breast cancer to evaluate whether alterations in this physiological pathway of cell elimination and tissue homeostasis may be a further mechanism giving breast cancer cells a survival advantage.

Material and Methods

Patients

103 tumour specimens and blood samples were obtained from premenopausal (n=30) and postmenopausal (n=73) women with breast carcinoma treated at the Department of Oncology, Institute of Polish Mother's Memorial Hospital between 2002–2003. There were 67 carcinoma ductale, 14 carcinoma lobulare and 22 other histological types of cancer (papillary, lobular, mucinous). The patients ranged in age from 32 to 79 years (mean age ± SD, 56 ± 10.97 years). The pathological evaluation report was obtained for each patient. According to the size of the tumor, the samples were divided into four categories: $T_1 \leq 2$ cm (62 tumors); T_2, 2–5 cm (29 tumors); T_3, > 5 cm (9 tumors); T_4 any size with direct extension to chest wall or skin (3 tumors). According to the appearance of metastasis in the axillary lymph nodes, the samples were allocated to four categories: N_0 (59 tumors), N_1 (26 tumors), N_2 (10 tumors) and N_3 (8 tumors). All tumors were classified as M_0. The average tumor size was 25 mm (range 50–100 mm). 67 samples of ductal breast carcinoma were graded by a method based on the criteria of Bloom-Richardson. There were 7 tumours of I° grade, 40 of II° grade and 20 of III° grade in total. The patients had not received chemo- or hormone therapy. For all tumors estrogen-receptor expression levels and progesteron-receptor expression levels were determined routinely by immunohistochemistry. Blood samples from age matched healthy women (n=90) served as control. The specimens were fixed in 10% formalin, embedded in paraffin wax, and 4 μm consecutive sections were used for histological examination and in situ detection of apoptotic cells.

TUNEL methods

Apoptotic cells were detected by the terminal deoxynucleotide transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL) method using commercially available ApopTaq Peroxidase In Situ Apoptosis Detection Kit (Intergen Company, USA) according to manufacturer's instruction.

Detection of apoptosis in peripheral blood cells

One of the features of apoptotic cells is fragmentation of chromatin DNA at nucleosome level (185 bp). Usually this fragmented DNA is detected with laddering in gel electrophoresis.

The genomic DNAs were extracted from peripheral blood samples collected in the morning in the presence of EDTA. The blood sample was treated with an erythrocyte lysis solution (155 mM NH4CL, 10 mM KHCO3 and 1 mM EDTA, pH 7.4) and then with extraction buffer (10 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K, pH 8.2) at 57°C, overnight. The DNA was precipitated with 1.5 M NaCl solution and ethanol. After washing with 70% ethanol, the preparation was resuspended in water and the DNA concentration was obtained by measuring absorbance at 260 nm. Three micrograms of the DNA was applied to a 1% agarose gel, using TBE (0.09 M Tris-borate, 2 mM EDTA, pH 8.3) as electrophoresis buffer. Electrophoresis was performed at approximately 5 V/cm. The gels were stained with 1 mg/ml ethidium bromide and viewed under UV light.

Statistical analysis

None of the parameters recorded in tumour material passed tests for being normally distributed (Smirnow-Kolmogorov test) and therefore nonparametric statistical tests were used for analysing the results. Statistical evaluation of differences in quantitative data between the two groups was performed using the Mann-Whitney U test. Spearman exact probability test was used to assess the associations between parameters and pathologic data. A P value less than 0.05 was considered to be statistically significant.

Results

Apoptosis in peripheral blood cells

Apoptosis was determined in 103 breast carcinoma samples and in 90 control samples. Apoptotic peripheral

TABLE 1
Number of patients presenting or not apoptotic peripheral blood cells as compared with controls

Patients (n=103)				Control (n=90)				P-value[a]
Apoptosis				Apoptosis				
Positive		Negative		Positive		Negative		
n	%	n	%	n	%	n	%	$P < 0.001$
72	69.9	31	30.1	1	1.1	89	98.9	

[a]as compared with controls

TABLE 2
The presence of apoptotic peripheral blood cells in patients with node-positive (n=44) and node-negative (n=59) breast cancer

	Node-positive breast cancer patients			
	Apoptosis			
	Positive		Negative	
	n	%	n	%
N_1	20[a]	76.9	6	23.1
N_2	7	70.0	3	30.0
N_3	7	87.5	1	12.5
total	34	77.2	10	22.7
	Node-negative breast cancer patients			
	Apoptosis			
	Positive		Negative	
	n	%	n	%
N_0	38	64.4	21	35.6

[a]P = 0.366 as compared with node-negative patients

TABLE 3
Number of breast cancer[a] patients presenting or not apoptotic peripheral blood cells in relation to tumour grade

grade[b]	I° (n=7)		II° (n=40)		III° (n=20)	
Apoptosis	Number	Frequency	Number	Frequency	Number	Frequency
Negative	3	0.43	12	0.30	4	0.20
Positive	4	0.57	28	0.70	16	0.80

[a]n = 67; [b]according to Bloom-Richardson criteria; [c]P=0.492

blood cells in all patients and healthy volunteers was detected by the agarose gel electrophoresis.

The presence of apoptosis in blood cells for breast cancer patients and control are shown in Table 1. 72 of 103 carcinoma samples (69.9%) were positive for the presence of apoptosis in blood cells and 1 of 90 control samples (1.1%) were positive for the presence of apoptotic peripheral blood cells. The presence of apoptosis in cancer samples was higher than in normal samples ($P < 0.001$). Among apoptosis-positive samples 76.7% (23/30) were from premenopausal and 67.1% (49/73) from postmenopausal women. Analysis of apoptosis in cancer samples obtained from premenopausal and postmenopausal women showed no differences (Mann-Whitney U test, P=0.470).

The studies of the presence of apoptosis in the peripheral blood cells for node-positive and node-negative breast cancer patients are displayed in Table 2. Apoptosis in patients varied widely depending on the lymph node status of the tumours. Of the 44 node-positive women, 34 (77.27%) exhibited apoptosis. In lymph node-negative tumours, the presence of apoptotic cells did not differ significantly (P=0.366) compared as lymph node-positive tumours; 38 (64.41%) node-negative subjects exhibited apoptosis.

Dependencies of the distribution of frequencies of apoptotic cells on the tumour grade evaluated according to Scarf-Bloom-Richardson criteria in women with ductal breast carcinoma are displayed in Table 3. There were no significant differences between apoptotic effect in subgroups assigned to histological grades ($P > 0.05$).

Apoptotic index (AI)

The apoptotic process is often described by means of the apoptotic index (AI), which may refer to the number of apoptotic cells per square milimetre of neoplastic tissue in the section. Table 4 shown the relationship between tumor size, histological type of cancer, sex steroid receptor status and AI. There were the significant differences between tumor size and apoptotic index. The large tumors (diameter >2 cm) have significantly higher AI than small tumors (diameter<2 cm) (P<0.001). In ductal breast carcinomas AI was

TABLE 4
Relationship between tumor size, histological category, sex steroid receptor status and apoptotic index (AI)

	No.	*P*-value[a]	AI
Tumor size			
≤2,0 cm (median 25–75%) >2 cm (median 25–75%)	62 41	*P*<0.001	11.0 (5.0–26.5) 26.0 (11.5–62.0)
Histological type			
– *carcinoma ductale* (median 25–75%) – *carcinoma lobulare* (median 25–75%)	67 14	*P*=0.111	18.0 (8.25–33.5) 8.0 (4.0–41.0)
– *carcinoma ductale* a) I° b) II° c) III°	7 40 20	*P*=0.001 *P*<0.001 *P*<0.001	4.0 (3.0–7.0) 14.0 (8.5–27.5) 40.0 (26.5–70.5)
Estrogen-receptor ER (+) (median 25–75%) ER (-) (median 25–75%) Progesteron-receptor PR (+) (median 25–75%) PR (-) (median 25–75%)	 64 39 61 42	 *P*=0.002 *P*=0.003	 11.5 (5.5–26.5) 25 (10.5–53.5) 12.0 (5.75–25.25) 27.5 (10.0–46.0)

[a]evaluation by Mann-Whitney U-test

higher than in the other histological types (lobular, papillary, mucinous). The differences were statistically significant (*P*=0.111). Moreover the significant relationship between sex steroid receptor negativity and high AI was detected. Tumors that express oestrogen receptor (ER) have lower AI than ER-negative tumors. The relationship between progesterone receptor (PR) and AI was similar. The sex steroid receptor content and AI show a statistically significant inverse relationship.

Discussion

Breast cancer is the commonest malignancy in women and comprises 18% of all cancers in women. In present work the apoptosis phenomenon and its role in the development and prognosis of breast cancer was investigated.

To determinate tumour behaviour, we must consider not only the proliferation, but also cell death. The growth rate of a tumour depends on both the proliferation and loss of tumour cells. Both necrosis and apoptosis induce cell death. Apoptotic cell death plays a important role in the pathogenesis and disease progression of cancer. The apoptotic process is controlled by inducers and repressors, the balance between these stimuli determining whether the cell cycle enters mitosis or apoptosis [2, 19].

With increased understanding of the physiological events that occur during apoptosis, a number of assay methods have been developed for its detection. These assays are based on different events which characterize the apoptotic process. Apoptosis and cell mediated cytotoxicity are characterized by a fragmentation of the genomic DNA. These DNA fragments have a length of about 180 base pairs or multiples there of (360, 540, 720, ...), the characteristic DNA-length of a nucleosome (DNA-histone-complex). Endonucleases selectively cleave DNA at sites located between nucleosomal units (linker DNA). In agarose gel electrophoresis these DNA fragments are resolved to a distinctive ladder pattern [3]. In this work we demonstrated that apoptosis might be successfully detected by agarose gel electrophoresis in blood samples.

In an attempt to determine if tumour cells from breast cancer are under control of apoptosis, genomic DNA was extracted from 103 blood samples obtained from patients diagnosed at the Polish Mother's Memorial Hospital. The patients included in the study received no chemotherapy or hormone therapy. DNA analysis by agarose gel electrophoresis using peripheral blood cells from the patients showed the characteristics pattern of DNA fragmentation. Fig. 1 represents an agarose gel electrophoresis of representative patients (panel A) and healthy volunteers (panel B), where the DNA fragmentation profile is quite clear. 69.9% breast cancer patients presented such profile which is a hallmark of the apoptosis process; while 1 of the blood samples from 90 healthy individuals (98.9%) showed this pattern. The observation of the apoptotic profile in patient blood cells suggests that these cells might be dying massively.

The relationship between lymph node status and apoptotic cell death was analysed according to either the

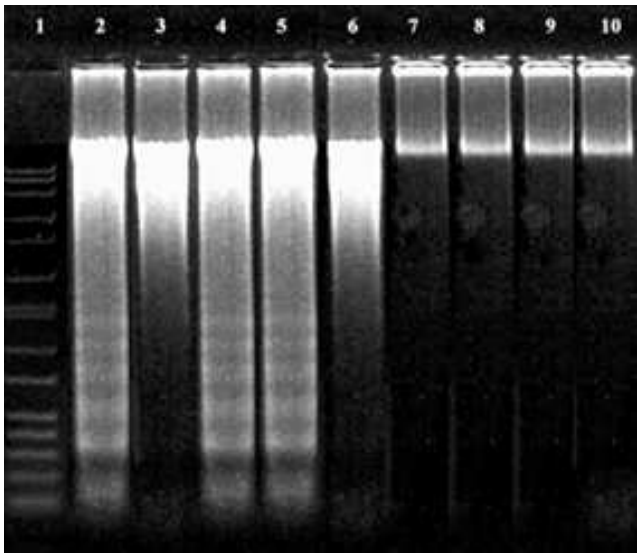

Fig. 1. Agarose gel electrophoresis of peripheral blood cell DNA obtained from breast cancer patients (panel A-lanes 2–6) and from control (panel B-lanes 7–10). Lane 1–100 bp ladder (Sigma-Aldrich, Germany).

presence or the number of involved lymph nodes. Most studies not found a statistically significant relationship between the presence of positive nodes and apoptotic cells [20]. Lipponen et al. [12] reported lack of a significant relationship between high AI and axillary lymph node involvement. In our study we did not find also a significant difference in the number of patients presenting apoptosis or not in relation to lymph node status.

The histological analysis of tumour grade showed a good relation between grade III tumours and the number of patients presenting apoptotic peripheral blood cells. 16 of 20 breast cancer patients with grade III° tumours were positive for the presence of apoptosis in blood cells. The grade corresponds to the tumour aggressiveness and is correlated with patient survival. Patients with grade I tumours have better survival than those with grade II and III.

Apoptosis can be detected in breast cancer by counting the apoptotic cells in conventional histopathological sections or by using special staining techniques, which are based on *in situ* labelling of the fragmented DNA (terminal deoxynucleotidyl transferase-mediated UTP nick end-label (TUNEL staining) [8, 13, 17]. The apoptotic process is often described by means of the apoptotic index (AI), which may refer to the number of apoptotic cells.

In our study that included 103 breast cancer patients and used TUNEL methodology the relationship between tumor size >2 cm and AI was present. Our study confirmed studies Zhang et al. [21], that large tumors have significantly higher AIs than small tumors.

In invasive ductal carcinomas, AIs are usually higher than in the other histological types (papillary, lobular, mucinous), but medullary carcinomas also show high AIs [12]. However, the findings are not consistent, as the AIs reported in some small studies have been about equaal in invasive lobular and invasive ductal carcinomas [13]. In our study ductal breast carcinomas AIs was higher than in the other histological types (lobular, papillary, mucinous).

Data relating AI to sex steroid receptor content are variable. Two large studies [1, 12] that included a total of more than 1000 breast carcinomas have established a significant relationship between sex steroid receptor negativity and high AI. Our studies confirmed that ER+ tumors have usually lower AIs than ER- tumors. The relationship between PR+ and PR- tumors and AI is similar.

Our findings provide additional evidence that genetic alterations, including apoptotic cell death, may occur as relatively early events in the development of breast cancer.

Our study implies that the apoptosis process is associated with breast cancer appearance and its invasiveness. Further studies, conducted on a larger population, are required to clarify this point.

Acknowledgements: This work was supported by grant 3P05C06923 and 3P05C06624 from the State Committee of Scientific Research (KBN).

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