Pol J Pathol 2003, 54, 1, 25-29 PL ISSN 1233-9687

Originals

Elżbieta Kozłowska[1], Hanna Romanowicz-Makowska[1], Marek Zadrożny[2], Joanna Mankiewicz[3], Krzysztof Szyłło[4], Tomasz Pertyński[5], Andrzej Kulig[1], Beata Smolarz[1]

Detection and Analysis of Apoptosis in Breast and Ovarian Cancer Subjects - Genetic Studies*

[1]Laboratory of Molecular Genetics, Department of Pathology,
[2]Department of Oncology, Institute of Polish Mother's Memorial Hospital,
[3]Department of Molecular Biology, University of Łódź,
[4]Department of Surgical Gynaecology,
[5]Department of Gynaecology and Menopausal Diseases, Institute of Polish Mother's Memorial Hospital, Łódź

Apoptotic cell death plays a central role in the pathogenesis and disease progression of cancer as well as in the response to treatment. In the present work we investigated the association between the presence of apoptotic peripheral blood cells in breast and ovarian cancer progression. Apoptosis was analysed in blood cells of breast (n=82) and ovarian cancer patients (n=79). Blood samples from age matched healthy women served as control (n=70). The apoptotic peripheral blood cells were detected by agarose gel electrophoresis. The apoptotic cells were identified in 83% (68/82) of the breast cancers and in 65% of ovarian cancer patients (51/79). The number of positive samples was significantly higher among cancer samples than among control samples ($p<0.05$). Additionally, there were significant differences ($p<0.05$) in the presence of apoptosis between subgroups assigned to histological stage. The high frequency of apoptotic peripheral blood cells in breast and ovarian tumours suggests a potential role of apoptosis in cancer appearance and/or progression.

Introduction

Apoptosis is a process whereby cells die in a controlled manner in response to specific stimuli and apparently according to an intrinsic and specific program [13]. This process plays an important role in cell-deletion in normal homeostasis and embryogenesis, and contributes to retardation of tumour growth [33].

Apoptosis process is a frequent phenomenon both in breast and in ovarian cancer [1, 7, 15, 19]. Ovarian cancer, one of the most common gynaecological malignancies, has an aggressive phenotype and a relatively poor prognosis; peritoneal dissemination and/or retroperitoneal lymph node metastases are found in two-thirds of patients at the time of diagnosis [5]. The ability of ovarian cancer cells to metastasise to different sites in the body is the major cause of morbidity and mortality among ovarian cancer patients. It is therefore important to identify high-risk or low-risk patients by suitable markers.

Breast cancer is one of the major killers worldwide. The most important prognostic parameters for breast carcinomas are tumour stage, histological subtype, grade, and residual tumour after surgical treatment [11, 18]. However, these factors present an incomplete picture of the tumour biology of breast cancer. Therefore, investigation of other prognostic factors is of special clinical relevance, particularly in view of the unexpectedly progressive course of the disease and frequent relapses in some cases.

In the regulation of tumour mass, a balance between rates of cell proliferation and cell loss is crucial. Cell loss by cell death from cancers has begun to receive attention as a possible indicator of tumour growth [33]. Among various malignant tissues examined, morphological evidence of apoptosis was commonly found and apoptosis demonstrated in regressing tumours [12, 13]. Moreover, it was reported that *in vitro* tumour cells can gain a selective growth advantage by blocking the apoptotic pathway [12, 13, 33]. These findings show that apoptosis is a mode of cell death in cancers and its frequency may serve as a useful variable to characterise the behaviour of tumours.

Dysregulation of the normal programmed cell death mechanism plays an important role in the pathogenesis and progression of ovarian [1, 2, 19, 35] and breast cancer [14, 19, 34].

The BCL-2 family with its anti-apoptotic members BCL-2, BCL-XL, MCL-1, and A1 and the growing subfamily of death-promoting members BAX, BCL-XS, BAK, BAD, BIK, BID, BIM, BOK, HRK, MTD, and BOO play a central role in the regulation of apoptotic cell death [23, 24,

*This work was supported by grant 3P05C06923 from the State Committee of Scientific Research (KBN)

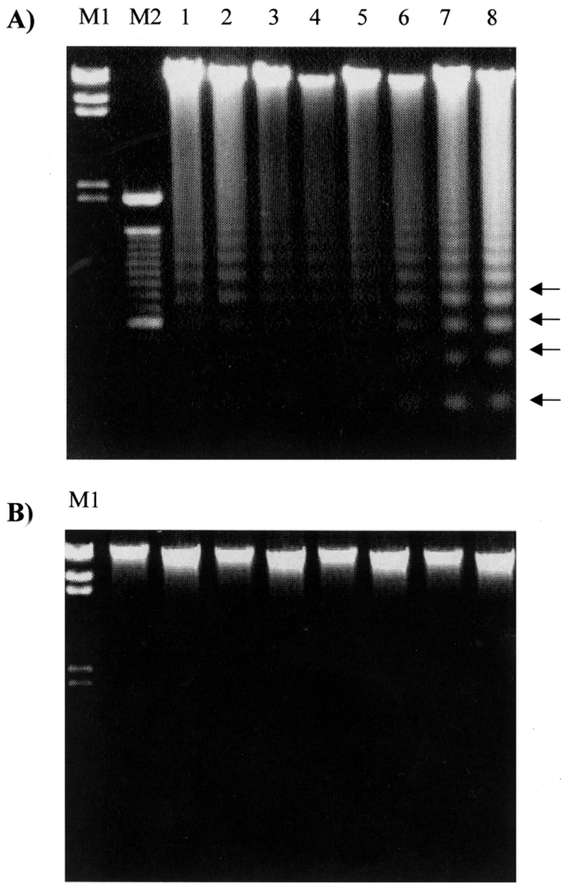

Fig. 1. Agarose gel electrophoresis of peripheral blood cell DNA obtained from breast and ovarian cancer patients (panel A) and from control (panel B). Note the characteristic apoptotic pattern present in the peripheral blood cells from breast cancer subjects in the lanes 1 - 4 and from ovarian cancer patients in the lanes 5 - 8 presented in panel A and indicated by the arrows. M1 - lambda-Hin*dIII* markers (Qiagen, Hilden, Germany), M2 - 100bp ladder (Qiagen, Hilden, Germany).

29, 37]. In breast cancer patients, reduced BAX expression correlates with a poor response to chemotherapy and shorter overall survival [16, 27, 28, 30]. BCL-2 expression represents an independent prognostic predictor in stage III ovarian cancer [1, 2].

In the present work, we addressed the question whether the frequency of apoptosis detected by agarose gel electrophoresis correlates with breast and ovarian cancer progression.

Material and Methods

Breast cancer samples

Blood was obtained from 82 women with node-negative (n=34) and node-positive (n=48) ductal breast carcinoma treated at the Department of Oncology of the Institute of Polish Mother's Memorial Hospital. No distant metastases were found in patients at the time of treatment. Blood samples were collected from 40 premenopausal women (mean age±SD 41.33±4.72 years) and from 42 postmenopausal women (mean age±SD 65.33±7.66 years). Median follow-up of patients still alive at the time of analysis was 39 months (range: 2 - 71 months). The average tumour size was 20mm (range 17 - 32mm). All tumours were graded by a method based on the criteria of Scarff-Bloom-Richardson [6, 25]. There were 27 tumours of I grade, 29 of II grade and 26 of III grade in total. Steroid receptor status was not determined in the study group.

Ovarian cancer samples

Blood samples of 79 postmenopausal women with ovarian cancer treated at the Department of Surgical Gynaecology of the Institute of Polish Mother's Memorial Hospital were studied. The patients ranged in age from 59 to 82 years (mean age±SD 66 .43±6.68 years). All tumours were staged according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO). There were 25 tumours of I stage, 30 of II stage and 24 of III stage in total.

Control samples

Blood samples from age matched healthy women (n=70) served as control.

DNA isolation

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 (155mM NH_4CL, 10mM $KHCO_3$ and 1mM EDTA, pH 7.4) and then with extraction buffer (10mM Tris-HCl, 100mM NaCl, 25mM EDTA, 0.5% SDS and 0.1mg/ml proteinase K, pH 8.2) at 57˚C, overnight. The DNA was precipitated with 1.5M 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 260nm. Three micrograms of the DNA was applied to a 1% agarose gel, using TBE (0.09M Tris-borate, 2mM EDTA, pH 8.3) as electrophoresis buffer. Electrophoresis was performed at approximately 5V/cm. The gels were stained with 1mg/ml ethidium bromide and viewed under UV light (Fig. 1).

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 to analyse the results. P-values <0.005 were considered as significant.

TABLE 1

The proportion of breast cancer patients presenting or not apoptotic peripheral blood cells

Breast cancer patients (n=82)		Control (n=70)	
Apoptosis		Apoptosis	
Positive	Negative	Positive	Negative
68(0.83)[a]	14(0.17)	13(0.19)	57(0.81)

[a]p<0.05 as compared with controls

TABLE 2

The proportion of ovarian cancer patients presenting or not apoptotic peripheral blood cells

Ovarian cancer patients (n=79)		Control (n=70)	
Apoptosis		Apoptosis	
Positive	Negative	Positive	Negative
51(0.65)[a]	28(0.35)	13(0.19)	57 (0.81)

[a]*P*<0.05 as compared with controls

TABLE 3

The proportion of breast cancer[a] patients presenting or not apoptotic peripheral blood cells in relation to tumour grade

grade[b]	I (n=27)		II (n=29)		III (n=26)	
Apoptosis	number	frequency	number	frequency	number	frequency
Negative	13	0.48	17	0.59	9	0.35
Positive	14	0.52	12	0.41	17	0.65

[a]n=82; [b]according to Scarf-Bloom-Richardson criteria

TABLE 4

The proportion of ovarian cancer[a] patients presenting or not apoptotic peripheral blood cells in relation to tumour grade

grade[b]	I (n=25)		II (n=30)		III (n=24)	
Apoptosis	number	frequency	number	frequency	number	frequency
Negative	12	0.48	16	0.53	8	0.33
Positive	13	0.52	14	0.47	16	0.67

[a]n=79; [b]according to FIGO criteria

Results

Apoptosis was determined in 82 infiltrating ductal breast carcinoma samples, in 79 ovarian cancer samples and in 70 control samples. Apoptotic peripheral blood cells in all patients and healthy volunteers were detected by the agarose gel electrophoresis.

The presence of apoptosis in blood cells for breast cancer patients and control are shown in Table 1. Sixty-eight out of 82 carcinoma samples (83%) were positive for the presence of apoptosis in blood cells and 13 out of 70 control samples (19%) were positive for the presence of apoptotic peripheral blood cells. The presence of apoptosis in cancer samples was higher than in normal samples ($p<0.05$). Among apoptosis-positive samples 32 were from premenopausal and 36 from postmenopausal women. Analysis of apoptosis in cancer samples obtained from premenopausal and postmenopausal women showed no differences (Mann-Whitney U test, $p=0.051$).

The studies of the presence of apoptosis in the peripheral blood cells for ovarian cancer patients are displayed in Table 2. Of the 79 ovarian cancer subjects, 51(65%) exhibited apoptosis. In control samples, the presence of apoptotic cells was lower ($p<0.05$) than in tumours; 13(19%) control 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 breast cancer and according to FIGO criteria in ovarian cancer patients are displayed in Table 3 and 4, respectively. There were significant differences in the apoptotic effect between subgroups assigned to histological grades ($p<0.05$). The histological analysis of tumour grade showed a good relation between grade III tumours and the number of patients presenting apoptotic peripheral blood cells.

Discussion

Tissues undergoing modifications during embryogenesis, tumour development or nontumoral rebuilding are affected by cell proliferation and deletion, concomitant phenomena which are often selective. In the wake of the pioneering work of Kerr and Wyllie [12, 13], the importance of cell loss through apoptosis (active cell death) and necrosis (passive cell death) [8, 26] has been increasingly recognised, and the focus is now on the balance between cell proliferation and cell elimination. Apoptosis frequently occurs in human tumours and seems to be a significant component of the continuous cell loss that usually takes place during tumour formation [13].

The development of breast and ovarian cancer is associated with an accumulation of specific genetic alterations. These genetic changes affect malignant transformation of both dysregulation of cell proliferation and apoptosis. The inhibition of apoptosis in cancer may contribute to tumour growth and promote neoplastic progression [30].

The apoptotic process can be triggered by an intracellular mechanism that seems to be dependent on the p53 protein, or by external factors such as hormones, cytokines and anti-tumour drugs. p53 can act as transcriptional regulator of the *BAX* gene, and part of the tumour-suppressor properties of the *p53* gene can be mediated by transcriptional activation

of the *BAX* gene [16, 20, 21, 22]. In breast cancer a defect in expression of the *BAX* mRNA and the BAX protein is a key promoter of apoptosis [4]. Restoration of BAX expression in breast cancer cell lines inhibited tumorigenicity [3] and increased sensitivity to cytotoxic drug therapy [32, 36]. In this line, reduced BAX expression was shown to be a negative prognostic factor in diffuse aggressive non-Hodgkin lymphoma [10], ovarian cancer [31] and pancreatic cancer [9]. p53, and especially the combination of p53 and BCL-2 expression data, represents an independent prognostic predictor in stage III ovarian cancer. Despite their role in the apoptotic process, p53 and BCL-2 do not seem to be clinically useful predictors of response to combination chemotherapy in these patients [2].

Apoptotic cell death plays a central role in the pathogenesis and disease progression of cancer. In an attempt to determine if tumour cells from breast and ovarian cancer are under control of apoptosis, genomic DNA was extracted from 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 characteristic pattern of DNA fragmentation. Figure 1 represents an agarose gel electrophoresis of representative patients (panel A) and healthy volunteers (panel B), where the DNA fragmentation profile is quite clear. Eighty--three per cent of breast cancer patients and 65% of ovarian cancer patients presented such profile, which is a hallmark of the apoptosis process; while 13 of the blood samples from 70 healthy individuals (19%) showed this pattern. The observation of the apoptotic profile in patient blood cells suggests that these cells might be dying massively.

The histological analysis of tumour grade showed a good relation between grade III tumours and the number of patients presenting apoptotic peripheral blood cells. Seventeen out of 26 breast cancer patients with grade III tumours were positive for the presence of apoptosis in blood cells (Table 3); 16 out of 24 ovarian 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.

The detection of apoptosis by gel electrophoresis initially involved the use of static gel electrophoresis for assessing DNA fragmentation to nucleosomes and nucleosome multimers [8]. In this work we demonstrated that apoptosis might be successfully detected by agarose gel electrophoresis in blood samples.

Recent studies have suggested that genetic alterations, including *p53* mutations, loss of heterozygosity, and microsatellite instability, can be detected in breast and ovarian cancer [17]. Our findings provide additional evidence that genetic alterations, including apoptotic cell death, may occur as relatively early events in the development of breast and ovarian cancer. Our study implies that it is possible that the apoptosis process may be involved in the appearance and/or progression of both breast and ovarian cancers. Further studies, conducted on a larger population, are required to clarify this point.

References

1. *Baekelandt M, Holm R, Nesland JM, Trope CG, Kristensen GB:* Expression of apoptosis-related proteins is an independent determinant of patient prognosis in advanced ovarian cancer. J Clin Oncol 2000, 18, 3775-3781.
2. *Baekelandt M, Kristensen GB, Nesland JM, Trope CG, Holm R:* Clinical significance of apoptosis related factors p53, Mdm2, and Bcl-2 in advanced ovarian cancer. J Clin Oncol 1999, 17, 2061.
3. *Bargou RC, Daniel PT, Mapara MY, Bommert K, Wagener C, Kallinich B, Royer HD, Dorken B:* Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis. Int J Cancer 1995, 60, 854-859.
4. *Bargou RC, Wagener C, Bommert K, Mapara MY, Daniel PT, Arnold W, Dietel M, Guski H, Feller A, Royer HD, Dorken B:* Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumour growth in SCID mice. J Clin Invest 1996, 97, 2651-2659.
5. *Berek JS, Fu YS, Hacker NF:* Ovarian cancer. In: Novak's Gynecology. Berek JS, Adashi EY, Hillard PA, eds. Williams & Wilkins, Baltimore 1996, 1155-1230.
6. *Bloom HJG, Richardson WW:* Histological grading and prognosis in breast cancer. A study of 1,409 cases of which 359 have been followed for 15 years. Br J Cancer 1957, 11, 359-377.
7. *Codegoni AM, Bertoni F, Colelle G, Caspani G, Grassi L, D'Incalci M, Broggini M:* Microsatellite instability and frameshift mutations in genes involved in cell cycle progression or apoptosis in ovarian cancer. Oncol Res 1999, 11, 297-301.
8. *Compton MM:* A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev 1992, 11, 105-109.
9. *Friess H, Lu Z, Graber HU, Zimmermann A, Adler G, Korc M, Schmid RM, Buchler MW:* Bax but not bcl-2 influences the prognosis of human pancreatic cancer. Gut 1998, 43, 414-421.
10. *Gascoyne RD, Krajewska M, Krajewsky S, Connors JM, Reed JC:* Prognostic significance of BAX protein expression in diffuse aggressive Non-Hodgkin's lymphoma. Blood 1997, 90, 3173-3178.
11. *Hortobagyi GN:* Treatment of breast cancer. N Engl J Med 1998, 339, 974-984.
12. *Kerr JF, Winterford CM, Harmon BV:* Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994, 73, 2013-2026.
13. *Kerr JFR, Wyllie AH, Currie AR:* Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26, 239-257.
14. *Krajewski S, Krajewska M, Turner BC, Pratt C, Howard B, Zapata JM, Frenkel V, Robertson S, Ionov Y, Yamamoto H, Perucho M, Takayama S:* Prognostic significance of apoptosis regulators in breast cancer. Endocrinol Relat Cancer 1999, 6, 29-40.
15. *Lipponen P:* Apoptosis in breast cancer: relationship with other pathological parameters. Endocrinol Relat Cancer 1999, 6, 13-16.
16. *Maciorowski Z, Klijanienko J, Padoy E, Mosseri V, Dieras V, El-Naggar AK, Chevillard S, Vielh P:* Differential expression of Bax and Bcl-2 in the assessment of cellular dynamics in fine-needle samples of primary breast carcinoma. Cytometry 2000, 42, 264-269.

17. *Malamou VD, Krikoni OC, Agnantis NJ:* Clonal analysis by PCR and RFLP in breast cancer and precancerous lesions, preliminary data. Anticancer Res 1996, 16, 3943-3948.
18. *McGuire W, Clark GM:* Prognostic factors and treatment decisions in axillary node-negative breast cancer. N Engl J Med 1992, 326, 1756-1761.
19. *Mendez O, Manas S, Peinado, Fabra A, Escobedo A, Moreno A, Sierra A:* Microsatellite instability is associated with the loss of apoptosis in ductal breast carcinomas. Breast Cancer Res Treat 2001, 65, 171-177.
20. *Miyashita T, Reed JC:* Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995, 80, 293-299.
21. *Morsi HM, Leers MP, Radespiel-Troger M, Bjorklund V, Kabarity HE, Nap M, Jager W:* Apoptosis bcl-2 expression and proliferation in benign and malignant endometrial epithelium: An approach using multiparameter flow cytometry. Gynecol Oncol 2000, 77, 11-17.
22. *Ouyang H, Furukawa T, Abe T, Kato Y, Horii A:* The BAX gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach and endometrium. Clin Cancer Res 1998, 4, 1071-1074.
23. *Reed JC:* Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994, 124, 1-6.
24. *Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime S, Bodrug S, Kitada S, Hanada M:* BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 1996, 60, 23-32.
25. *Scarf RW, Torloni H:* Histological Typing of Breast Tumors. Second edition. Geneva: World Health Organization 1981, 91-105.
26. *Searle J, Kerr JF, Bishop CJ:* Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annu 1982, 17, 229-259.
27. *Shilkaitis A, Graves J, Mehta RR, Hu L, You M, Lubet R, Steele V, Kelloff G, Christov K:* BCL-2 and BAX are differentially expressed in hyperplastic, premalignant, and malignant lesions of mammary carcinogenesis. Cell Growth Differ 2000, 11, 437-445.
28. *Shilkaitis A, Graves J, Mehta RP, Hu L, You M, Lubert R, Steele V, Kelloff G, Christov K:* Bcl-2 and Bax are differentially expressed in hyperplastic, premalignant, and malignant lesions of mammary carcinogenesis. Cell Growth Differ 2000, 11, 437-445.
29. *Song Q, Kuang Y, Divit VM, Vincenz C:* BOO, a novel negative regulator of cell death, interacts with Apaf-1. EMBO J 1999, 18, 167-178.
30. *Sturm I, Papadopoulos S, Hillebrand T, Benter T, Luck HJ, Wolff G, Dorken B, Daniel PT:* Impaired BAX protein expression in breast cancer: mutational analysis of the BAX and the p53 gene. Int J Cancer 2000, 87, 517-521.
31. *Tai YT, Lee S, Niloff E, Weisman C, Strobel T, Cannistra SA:* BAX protein expression and clinical outcome in epithelial ovarian cancer. J Clin Oncol 1998, 16, 2583-2590.
32. *Wagener C, Bargou RC, Daniel PT, Bommert K, Mapara MY, Royer HD, Dorken B:* Induction of the death-promoting gene bax-alpha sensitizes cultured breast cancer cells to drug-induced apoptosis. Int J Cancer 1996, 67, 138-141.
33. *Wyllie AH:* Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer and Metastasis Reviews 1992, 11, 95-103.
34. *Vakkala M, Lahteenmaki K, Raunio H, Paakko P, Soini Y:* Apoptosis during breast carcinoma progression. Clin Cancer Res 1999, 5, 319-324.
35. *Villa R, Folini M, Perego P, Supino R, Setti E, Daidone MG, Zunino F, Zaffaroni N:* Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: correlation with sensitivity to DNA damaging. Int J Oncol 2000, 16, 995-1002.
36. *Yin C, Knudson CM, Korsmeyer SJ, Van DT:* Bax suppresses tumorigenesis and stimulates in vivo. Nature 1997, 385, 637-640.
37. *Zhang GJ, Kimijima I, Watanabe T, Kanno M, Sagara H, Furukawa Y, Tsuchiya A, Abe R:* Correlation between apoptotic index, bcl-2 protein expression and progression and prognosis in breast carcinoma. Gan To Kagaku Rycho 1998, 25, 415-421.

Address for correspondence and reprint requests to:
B. Smolarz M.D.
Laboratory of Molecular Genetics
Department of Pathology
Institute of Polish Mother's Memorial Hospital
Rzgowska 281/289, 93-338 Łódź
fax: +48-42 271 14 21