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Research article

Cell culture conditions potentiate differences in the response to ionising radiation of peripheral blood leukocytes isolated from breast cancer patients and healthy subjects

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To compare the effects of ionising radiation on leukocytes from breast cancer patients and healthy subjects *ex vivo*, the level of NF-κB and the antioxidant enzymes manganese-containing superoxide dismutase (Mn-SOD), copper/zinc-containing superoxide dismutase (CuZn-SOD) and catalase (CAT) in combination with flow cytometric analysis of $CD4^+$ lymphocytes was performed. The level of Mn-SOD protein was significantly increased in the breast cancer study group both before ($P < 0.001$) and after ($P < 0.001$) irradiation when compared with healthy subjects. Measurements in parallel indicated that the level of CAT protein was significantly higher in the breast cancer study group after irradiation (2 Gy [$P < 0.001$] and 9 Gy [$P < 0.05$]) when compared with healthy subjects. Although the initial number of lymphocytes in the blood of breast cancer patients was not different from healthy subjects, the percentage of apoptotic $CD4^+$ cells was significantly ($P < 0.001$) lower both before and after irradiation indicating that cell culture conditions induced radioresistance of $CD4^+$ cells in the blood of breast cancer patients. The data presented in this current study indicate that brief *ex vivo* culture of peripheral blood leukocytes potentiates oxidative stress imposed by a breast cancer tumour.

Keywords: Ionising radiation, leukocytes, breast cancer, superoxide dismutase, catalase

INTRODUCTION

Breast cancer is the most common form of malignancy in women and is characterised by hormonal, genetic and environmental origins.[1] Certain forms of breast cancer constitutively produce substantial quantities of reactive oxygen species (ROS) such as the superoxide anion radical, the hydroxyl radical and hydrogen peroxide (H_2O_2) that confer a potential advantage in proliferation, migration and invasion of surrounding tissues through the degradation of the extracellular matrix and an increase in cell motility.[2–4] Furthermore, the role of ROS in the spread of breast cancer may not only be limited to their mutagenic activity that drives carcinoma progression.[5] In our previous study, we demonstrated that breast cancer also created systemic conditions that increased the level of H_2O_2 in circulating cells.[6] Such systemic conditions may also favour survival and further invasion of breast cancer cells.

Many features of breast cancer are clinically treatable by chemotherapy and/or external irradiation.[7] Administration of selected chemotherapy agents (including cyclophosphamide and doxorubicin) and ionising radiation results

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in free radical and ROS production intended to reduce the rate of breast cancer cell proliferation.[8] Unfortunately, a number of the physiological side effects of these therapies are related to oxidative damage induced by increased ROS.[9,10] We have previously shown that breast cancer CAF chemotherapy (a combination of cyclophosphamide, doxorubicin and fluorouracil) failed to compensate for the increase in H_2O_2, therefore creating systemic conditions for the survival and invasion of breast cancer cells.[6] It is, therefore, suggested that the modulation of the oxidant/antioxidant balance towards a more reduced state by the systemic administration of low molecular weight substances such as selenium may limit invasion of most cancer cells and relieve the side effects of CAF therapy. For the evaluation of the systemic effect of ROS imposed by either breast cancer or by anti-cancer and/or antioxidant supplementation therapy, a simple clinical *ex vivo* test would be convenient. One possible candidate for such a test system might be a versatile and rapid assay that has been previously used to determine the sensitivity to ionising radiation in healthy individuals based on evaluation of peripheral blood leukocyte cytotoxicity.[11,12] The quantification of apoptotic $CD4^+$ lymphocytes, the highly ionising radiation-sensitive subpopulation of peripheral blood leukocytes, is performed by flow cytometric evaluation of their size, granularity, antigen immunofluorescence and DNA content. It was shown to be a highly sensitive biological 'dosemeter' capable of detecting exposures as low as 0.05 Gy with discrimination of ± 0.1 Gy.[13]

In the present study, we performed experiments to determine if differences in NF-κB, manganese-containing superoxide dismutase (Mn-SOD), copper/zinc-containing superoxide dismutase (CuZn-SOD) and catalase (CAT) expression persisted in peripheral blood cells after primary culture and if the Crompton's lymphocyte cytotoxicity assay may be used to evaluate the effects of a further increase in the level of ROS. We used ionising radiation as a source of ROS (ionising radiation is known to generate ~10^{-6} M of different ROS per 1-Gy dose while the cumulative intracellular concentration of ROS in healthy tissues is ~10^{-8} M[14,15]).

Patients and Methods

Patients

Sixteen women diagnosed with breast cancer (age range, 30–60 years; mean, 49.9 ± 9.7 years) were recruited for this study prior to receiving any medication. The patients were clinically categorised as stages III or IV breast cancer and locally advanced breast cancer (LABC) patients. Sixteen women deemed healthy (age range, 30–60 years; mean, 44.8 ± 8.4 years) were used as control subjects. Both breast cancer/LABC patients and healthy controls were fully informed and consented to be volunteers in our study. The whole study was approved by the Institution Review Board of the Institute of Oncology and Radiology of Serbia.

Cell irradiation and flow cytometry analysis

Heparinised whole blood samples were diluted 1:10 in RPMI 1640 medium with 20% fetal bovine serum before being divided into three 25-cm^2 flasks (5 ml per flask). One flask was left as a control whereas the other two were irradiated with 2 Gy and 9 Gy (the gamma ray dose rate was 20 Gy/h). Thereafter, the flasks were placed in a culture incubator for 48 h at 37°C in an atmosphere of 5% CO_2/95% air. Blood cells (4.5 ml of the suspension) were pelleted by centrifugation (200 *g* for 10 min at room temperature) before being lysed (see below). The remaining 400 µl of suspension was incubated for 15 min at room temperature in the dark with a monoclonal anti-CD4 antibody (Becton Dickinson, BD#345768; CD4-FITC, 10 ml per 200 ml of cell suspension). Erythrocytes were lysed using FACS lysing solution (BD#349202). Leukocytes were pelleted, washed with PBS and resuspended in 0.1 ml of FACS Flow solution (BD#342003) followed by the addition of 0.05 ml of RNase (bovine pancreas RNaseA; Serva, Heidelberg, Germany). Propidium iodide was added (final concentration 1 mg/ml) to stain the cellular DNA. Multiparameter sample measurements were performed using a BD FACScalibur flow cytometer to discriminate the three major types of leukocytes and to gate for normal and apoptotic lymphocytes allowing data acquisition.[11] The data were processed with BD Lysis II software.

SDS-PAGE and Western blotting

Cell pellets from whole blood samples (see above) were lysed using 10 mM Tris-HCl pH 7.4 buffer containing 1% Triton X-100, 0.32 M sucrose, 5 mM $MgCl_2$ and protease inhibitor cocktail The protein concentration was determined by the method of Lowry *et al*.[16] Proteins in aliquots of denatured lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[17] before being transferred to nitrocellulose membranes. The membranes were blocked using 10 mM Tris buffer pH 7.4 containing 150 mM NaCl, 1% BSA and 0.1% Tween-20. The membranes were separately incubated overnight (at 4°C) with the following antibodies: rabbit anti-NF-κB (NF-κB p65 C-20; SC-372; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Mn-SOD and rabbit anti-CuZn-SOD (SOD-101, SOD-102

Table 1. Protein expression of NF-κB, Mn-SOD, CuZn-SOD and CAT in the blood cells of healthy subjects and breast cancer patients treated with 0, 2 or 9 Gy of ionising radiation (represented as mean ± SD)

Group	NF-κB level (AU/mg)	Mn-SOD level (AU/mg)	CuZn-SOD level (AU/mg)	CAT level (AU/mg)
Control (n = 16)	9.0 ± 4.5	55.1 ± 26.3	41.1 ± 7.1	6.7 ± 3.3
Control + 2 Gy	7.5 ± 4.4	59.6 ± 27.8	43.4 ± 8.9	5.8 ± 2.9
Control + 9 Gy	7.2 ± 3.6	60.5 ± 33.6	43.5 ± 11.0	5.3 ± 3.4
Breast cancer (n = 16)	8.3 ± 4.7	92.3 ± 45.1***	46.1 ± 8.6	9.1 ± 5.1
Breast cancer + 2 Gy	7.9 ± 4.9	113.7 ± 86.8***	46.0 ± 5.5	12.2 ±7.3***
Breast cancer + 9 Gy	7.8 ± 5.0	126.8 ± 99.3***	43.5 ± 7.1	8.1 ± 5.2*

Significant differences (***P < 0.001) between breast cancer samples (non-irradiated and irradiated) and their corresponding control samples were apparent in the case of Mn-SOD. Significant differences between breast cancer + 2 Gy and control + 2 Gy and between breast cancer + 9 Gy and control + 9 Gy were observed in the case of CAT (***P < 0.001 and *P < 0.05, respectively).

Nventa [previously Stressgen Biotechnologies] San Diego, CA, USA), rabbit anti-CAT (Calbiochem, Darmstadt, Germany) and rabbit anti-actin (C-11: sc-1615; Santa Cruz Biotechnology). A secondary goat anti-rabbit IgG alkaline phosphatase conjugate SAB-301 (Stressgen Biotechnologies) was used for detection purposes. The quantification of specific antigen bands was achieved by computer image processing. The level of a specific protein was normalised to the level of actin and expressed in arbitrary units (AU/mg of total protein) from four independent Western blots. Statistical analyses were performed by using two-factor analysis of variance (ANOVA) according to previously published protocols.[18,19]

Results

Our study employed the conventional peripheral blood leukocyte radiosensitivity assay based on DNA and cell-surface marker assessment of cytotoxicity in order to monitor and evaluate the constitutive and radiation-induced level of the redox-sensitive protein NF-κB and the levels of the antioxidant enzymes Mn-SOD, CuZn-SOD and CAT in the blood cells of healthy subjects and breast cancer patients *ex vivo*.

The number of total lymphocytes, the number of $CD4^+$ lymphocytes and the percentage of apoptotic $CD4^+$ lymphocytes in blood samples taken from breast cancer patients and healthy subjects both before and after irradiation is shown in Figure 1 (A–C, respectively). The number of total lymphocytes in both study groups was not different. A significant reduction in the number of total lymphocytes was observed after 9 Gy irradiation only in the control group (Fig. 1A and Table 2). After irradiation, the fraction of $CD4^+$ lymphocytes in blood samples taken from breast cancer patients was significantly higher compared with that in healthy subjects (Fig. 1B and Table 2). The percentage of apoptotic $CD4^+$ lymphocytes was significantly lower in blood samples taken from breast cancer patients when compared with healthy subjects (Fig. 1C and Table 2). In addition, the

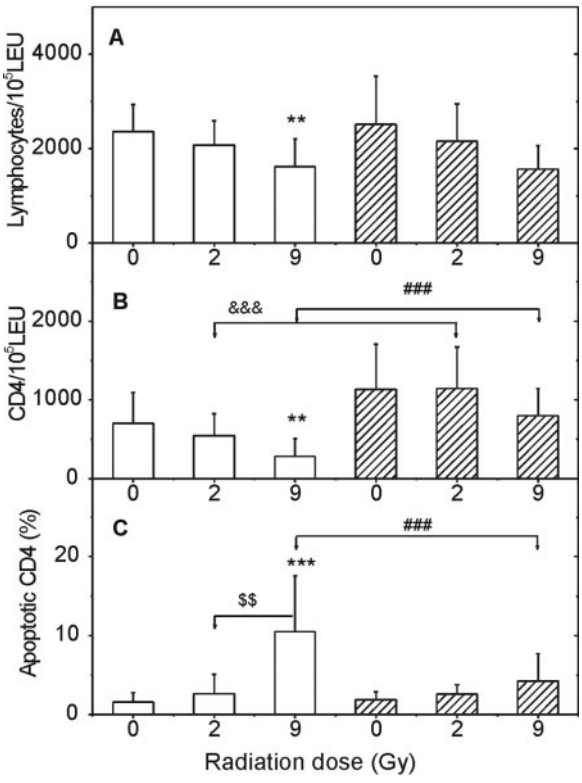

Fig. 1. The number of total lymphocytes (A), the fraction of $CD4^+$ lymphocytes per 10^4 leukocytes (B), and the percentage of apoptotic $CD4^+$ lymphocytes (C) in the blood of breast cancer patients and healthy subjects treated with 0, 2 or 9 Gy of ionising radiation (represented as mean ± SD). White bars, healthy subjects; hatched bars, breast cancer patients. In healthy subjects, significant differences between 9-Gy irradiated versus non-irradiated samples were apparent (***P < 0.001 and **P < 0.01). Significant differences (***P < 0.001 and *P < 0.05) between other measured samples are indicated by the horizontal links between the columns.

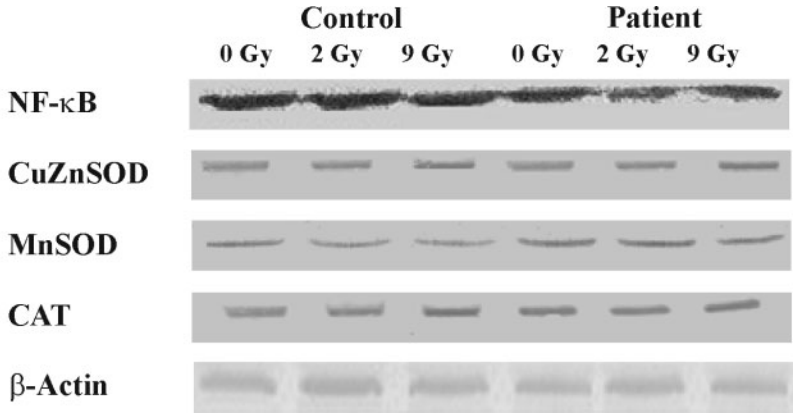

Fig. 2. Western blotting detection of NF-κB (p65), Mn-SOD, CuZn-SOD, CAT and β-actin in total blood cell lysates of breast cancer patients and healthy subjects treated with 0, 2 or 9 Gy of ionising radiation. Representative blots from repeated experiments are shown.

percentage of apoptotic $CD4^+$ cells significantly increased in blood samples taken from healthy subjects after irradiation (Fig. 1C and Table 2).

Western blotting of blood cell extracts revealed that the level of NF-κB protein was not significantly altered after either a 2-Gy or 9-Gy dose of irradiation in either of the two study groups (Fig. 2 and Table 1). The level of both Mn-SOD and CAT was significantly increased in blood samples of breast cancer patients both before and after irradiation when compared with healthy subjects (Fig. 2, Tables 1 and 2). The level of CuZn-SOD did not vary between blood samples of the two study groups.

Table 2. Two-way ANOVA of the measured parameters

		Disease (D)	Irradiation (I)	D x I	Error
CuZn-SOD	Df	1	2	2	111
	MS	187	17.7	60.8	66.3
	F	2.82	0.27	0.92	
Mn-SOD	Df	1	2	2	123
	MS	10,010	5370	2991	3613
	F	30.5***	1.49	0.83	
CAT	Df	1	2	2	109
	MS	486	34.2	40.6	22.3
	F	21.8***	1.53	1.82	
NF-κB	Df	1	2	2	105
	MS	2.65	16.8	3.62	20.3
	F	0.13	0.83	0.18	
LY	Df	1	2	2	54
	MS	43,495	3,515,262	59,367	438,524
	F	0.99	8.02***	0.14	
$CD4^+$	Df	1	2	2	54
	MS	3,819,858	767,646	31,688	152,609
	F	25.03***	5.03**	0.208	
Non-apoptotic cells	Df	1	2	2	50
	MS	0.006	0.027	0.017	0.0011
	F	6.07*	25.22***	15.89***	

Comparison factors are the presence of disease (D) and the irradiation (I). The analysis for non-apoptotic cells used logarithmic data values. The presence of disease significantly affected the activities of Mn-SOD and CAT. Irradiation significantly affected the number of lymphocytes (LY) and the $CD4^+$ population. Df, degrees of freedom; MS, mean square; F, values. Statistical differences (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$) refer to variable listed (CuZn-SOD, Mn-SOD, CAT, NF-κB, LY, $CD4^+$ and non-apoptotic cells) in the respective column (D, I or the combination D x I).

Discussion

The intracellular balance between ROS generation and ROS elimination, generally termed 'cellular redox homeostasis', requires co-ordination of several reactions (taking place within different cell compartments) performed by a complex network of pro-oxidant and antioxidant systems.[20] The latter consists of non-enzymatic ROS scavengers and detoxifying enzymes, as well as various redox-sensitive transcription factors such as NF-κB, p53 and AP1 that regulate oxidative stress-related gene expression.[21] Failure to limit ROS accumulation often results in cellular conditions promoting DNA damage, mutations and chromosomal aberrations which frequently leads to cell cycle arrest and cell death.[3–5] In addition, failure may also promote carcinogenesis.[22] A growing body of evidence suggests that some cancer cells exhibit increased intrinsic ROS stress, due to increased metabolic activity and mitochondrial malfunction.[23] In some situations, such conditions provide the cells with a potential advantage for proliferation, migration and invasion of the surrounding tissues.[2–4] Many of the currently used therapeutic strategies using cytostatic drugs and/or ionising radiation take advantage of the increased level of ROS in cancer cells to enhance therapeutic efficiency and selectivity.[21] The beneficial effects of these agents on tumour cell killing is also based on their ability to generate ROS and damage cells beyond their repair capacity. Both cytostatic drugs and ionising radiation frequently exhibit profound toxic side effects leading to healthy tissue damage,[21] such as those observed after breast cancer therapy.[6,24,25] Moreover, they may create systemic pro-oxidant conditions as described in our previous study.[6]

In our present study, the level of the redox-sensitive transcription factor NF-κB in combination with the level of the antioxidant enzymes Mn-SOD, CuZn-SOD and CAT may represent a convenient and easily available biomarker system for evaluating shifts in the redox potential towards oxidation imposed by the presence of malignant disease (such as breast cancer). The expression level of NF-κB was considered as an indicator of cell activation,[26] while Mn-SOD,[27] CuZn-SOD and CAT expression illustrated the ROS detoxification capacity of blood cells. We assumed that Crompton's assay would accurately detect ROS differences between breast cancer patients and healthy subjects *ex vivo*, as well as the response to ROS enhancement by ROS challenge *ex vivo*. For an *ex vivo* oxidative source, we decided to use ionising radiation as its dose is precisely controlled. We selected the doses of 2 Gy and 9 Gy, to be representative of the 1.8–2 Gy daily clinical doses given during curative radiotherapy and the 8–10 Gy single dose given in palliative radiotherapy. They correspond to 2 x 10^{-6} M and 9 x 10^{-6} M cumulative concentrations of various ROS.[14,15] Although the initial number of lymphocytes in the blood of breast cancer patients was not different from the number found in the blood isolated from healthy subjects, the $CD4^+$ lymphocyte subpopulation was increased in the blood from breast cancer patients. An increased $CD4^+$ subpopulation of lymphocytes has been previously connected with conditions of inflammation such as those observed in ATM syndrome and in Fanconi anaemia.[28] The fact that the percentage of apoptotic $CD4^+$ cells was significantly greater both before and after irradiation in the blood from healthy subjects indicated cell culture-induced radioresistance of $CD4^+$ cells in the blood of breast cancer patients. This radioresistance may have been the consequence of a significantly higher level of Mn-SOD and CAT.[29,30] In addition, radioresistance of $CD4^+$ lymphocytes in a population deemed healthy is related to donor age (the apoptotic response decreases linearly, 3% every 5 years, between 21–73 years of age). Thus, systemic exposure to endogenous breast cancer-induced ROS *in vivo* results in a $CD4^+$ profile characteristic of that found in a 10–15 year older healthy population.[31]

Our results indicated that NF-κB expression (when analysed 48 h after culture) in peripheral blood cells isolated from breast cancer patients was not different from that in cells isolated from healthy subjects. However, a difference in NF-κB expression in peripheral blood cells isolated from both breast cancer patients and healthy subjects at the moment of cell seeding has been previously reported by ourselves.[6] The current result indicates that NF-κB expression was attenuated by *ex vivo* cell culture conditions. In contrast to NF-κB, Mn-SOD expression (after 48 h of culture) in the blood cells of breast cancer patients still remained significantly higher compared to that in cells from healthy subjects. Elevated Mn-SOD expression accompanied by increased CAT expression in breast cancer blood cells may, therefore, represent a key survival factor, under the cell culture conditions. Oxidative stress clearly influences cell growth under culture conditions and this phenomenon has been recognised previously.[32] Our results show differential effects of cell culture on lymphocytes isolated from breast cancer patients and healthy subjects. The expression pattern of NF-κB, CuZn-SOD, Mn-SOD and CAT in blood cells from breast cancer patients was not significantly changed after either 2-Gy or 9-Gy doses of ionising radiation indicating that cell culture-induced oxidative stress made the cells more radioresistant.

Conclusions

The data presented in this current study indicate that brief culture of peripheral blood cells clearly potentiates the oxidative stress imposed by a breast cancer tumour.

Although the pattern of biomarker expression was slightly altered by *ex vivo* conditions, the degree of $CD4^+$ lymphocyte apoptosis may illustrate the degree of acquired systemic resistance to ROS imposed by the presence of a tumour. In addition, the combined assay may be used to investigate substitutional regimens, in other words to titrate out exact concentrations of antioxidants until the complete reversal of both biomarker expression and $CD4^+$ sensitivity to ROS is achieved.

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