MANGANESE SUPEROXID DISMUTASE LEVEL IN BLOOD CELLS OF PATIENTS WITH BREAST CANCER

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ABSTRACT

The intracellular MnSOD levels were determined in peripheral blood cells obtained from two age groups of patients with breast cancer (BC): 30-45 year old patients (premenopausal, n=7, clinical stage 1 to 3) and 46-60 year old patients (peri- and post-menopausal women, n=12, clinical stage 3 or 4), at diagnosis, prior to any clinical treatment. The respective healthy women groups were used as controls. Blood cells were also irradiated *in vitro* with 2- and 9- Gy of gamma-ray radiation from ^{60}Co source. The MnSOD levels were determined by the specific immunostaining and quantified by the laser densitometry. The MANOVA analysis and Tukey post-hoc test indicated significantly higher MnSOD levels in both groups of BC patients in relation to the respective controls ($F=25.166$, $p<0.001$). The data indicated that the increased initial MnSOD levels in peripheral blood cells may be related to the presence of BC *i.e.* they may reflect the system response to the presence of malignant tumour. In addition to that, *in vitro* radiation challenge of blood cells indicate that MnSOD overexpression may be a good indicator for selection of BC patients that would express increased resistance to oxidative stress imposed by the clinical treatment.

Key words: Breast cancer, Mn-superoxide dismutase, radioresistance

1.Introduction

The patients diagnosed with breast cancer (BC) are frequently exposed to clinical treatments with highly cytotoxic agents, such as ionizing radiation or cytostatic drugs, aiming to inhibit tumor cell proliferation and/or induce tumor cell death [1-3]. Ionizing radiation as well as cytostatic drugs generate high concentration of reactive oxygene species (ROS), which cause massive oxidative damage of tissue DNA, proteins and lipids [4]. The potential utilization and efficacy of radiation therapy for BC is limited by the necessity of avoiding excessive late damage to normal tissues induced by ROS [5]. The degree of healthy tissue damage determines the quality of life during the post therapy period. In order to protect them from excessive ROS induced damage, cells have evolved a spectrum of antioxidant defence enzymes. Among them is Mn superoxide dismutase (SOD2, EC1.15.1.1.) a mitochondrial enzyme (tetramer Mm of 88,000 g/mol) considered to be the first line in biological defence against cell damage by superoxide radicals. Mn superoxide dismutase (MnSOD) catalyzes the dismutation of superoxide radicals into H_2O_2 and O_2.

The overexpression of MnSOD both at the mRNA and protein level is corellated with increased radiation resistance [6-7], while the deficiency or low level enzyme activity of MnSOD are associated with the increased radiosensitivity [8]. Thus, the healthy tissue damage may be decreased by a local or a systematic application of protecting agents, such as *e.g.* antioxidants or purified antioxidant enzymes [9]. Aiming to adjust the treatment individually it is necessary to get the right measure of the endogenous oxidative damage repair capacity of each BC patient. In this study the expression levels of Mn SOD protein were determined in the peripheral blood cells of patients with BC and respective healthy women, in order to test if the enzyme expression may be a predictive biomarker of individual healthy tissue resistance to oxidative stress imposed by irradiation.

2. Experimental

a) Patients: The intracellular MnSOD levels were determined in peripheral blood of patients with breast cancer (BC), at diagnosis, prior to any clinical treatment. The assay was performed in the two groups of women with BC: age group 30-45 (premenopausal, n=7, clinical stage 1 to 3), and age group 46-60 (peri- and post-menopausal women, n=12, clinical stage 3 or 4). The respective healthy women: age group 30-45 (n=12) and age group 45-60 (n=12) were used as controls. Both patients and controls were informed consented. The study was approved by Institution Review Board at the Institute of Oncology and Radiology of Serbia.

b) Sample analysis: Aliquots of heparinized venous blood specimens were cultured in 10% RPMI after 2- and 9- Gy irradiation on ^{60}Co gamma-ray source at the dose rate of 20Gy/h. For total Mn SOD protein quantification erythrocytes and white blood cells were lysed by addition of Lysing Buffer containing 0.32M Sucrose, 10mM TrisHCl, 5mM MgCl2, and 1%Triton-100. For separate analysis of Mn SOD in erythrocyte or in the white blood cells, erythrocytes were lysed by addition of FACS^{TM} Lysing solution (Becton Dickinson). After centrifugation at 2,000 rpm for 5 min the supernatant was taken as a source of Mn SOD from erythrocytes, while the pellet was taken as a source of Mn SOD from white blood cells. White blood cells were lysed by repeated freeze-taw cycles. Samples (30 µl) of cell lysate were separateded on 10% polyacrylamide gels and transfered to a nitrocelulose membrane for Western blot analysis. For dot blot analysis the denatured blood cell extracts were applied directly to the nitrocellulose membrane using Manifold device (Schleicher and Schuell,Inc., Keene, NH, USA). The immunobloting was performed with Rabbit Anti–MnSOD Polyclonal Antybody (diluetid 1:3000). After washing the blots were incubated with alkaline phosphate-conjugated sheep anti rabbit antibody (Immuno–Reporter^{TM} Reagent DC05L, Oncogene Research Products, SanDiego, CA, USA) (diluted 1:4000). Immunoreactivity was detected by NBT/(5-bromo-4-chloroindol-3-yl phosphate, Sigma). The relative densities of the bands or dots was determined by UltroScan XL scanning laser densitometry and PC processing. The level of Mn SOD was expressed in arbitrary mass units (mean ± S.E.M., n=3-6 independent measurements). Multifactorial ANOVA (MANOVA) analysis was applied to estimate statistically relevant differences. If statistical significance was found, the Tukey post-hoc analysis was performed. Significance was accepted for $p<0.05$.

3. Results

In order to determine Mn SOD levels in peripheral circulation, total blood cell extracts, or erythrocyte and leukocyte extracts were resolved by denaturing 10% SDS-PAGE and immunostained by polyclonal rabbit anti-human Mn SOD antibodies. Mn SOD levels were determined either directly or after placing cells in the short term (48 h) primary culture in which they were challenged with 2 and 9 Gy of gamma radiation from ^{60}Co source. The typical WB profiles of Mn SOD in blood cell extracts from primary culture of two patient with breast carcinoma (BC) and respective control are shown in

Figure 1a. As could be seen the dominant Mn SOD bands in all samples were enzyme monomer with molar mass (Mm) *cca* 24 000 g/mol, and the second Mn SOD band with Mm of *cca* 50 000 g/mol. A smaller band with Mm *cca* 36 000 g/mol was also detected in some cases. The densitometric scanning of the bands showed increased leavel of MnSOD in all patient samples (Figure 1b, patient; K, 2Gy, 9Gy) compared to the respective controle samples (Figure 1b, control: K, 2Gy, 9Gy). Also, irradiation challenge of blood cells with 2Gy and 9Gy did not lead to marked changes in the level of Mn SOD either in the patients or control samples. When fresh blood cells (Figure 2, tot) were separated to erythrocyte (ery) and leukocyte (leu) fractions, the majority of Mn SOD was found in leukocyte fraction. It is necessary to note that in total cell and erythrocyte extracts MnSOD appeared as dimer, while leukocyte extracts contained Mn SOD in a form of tetramere.

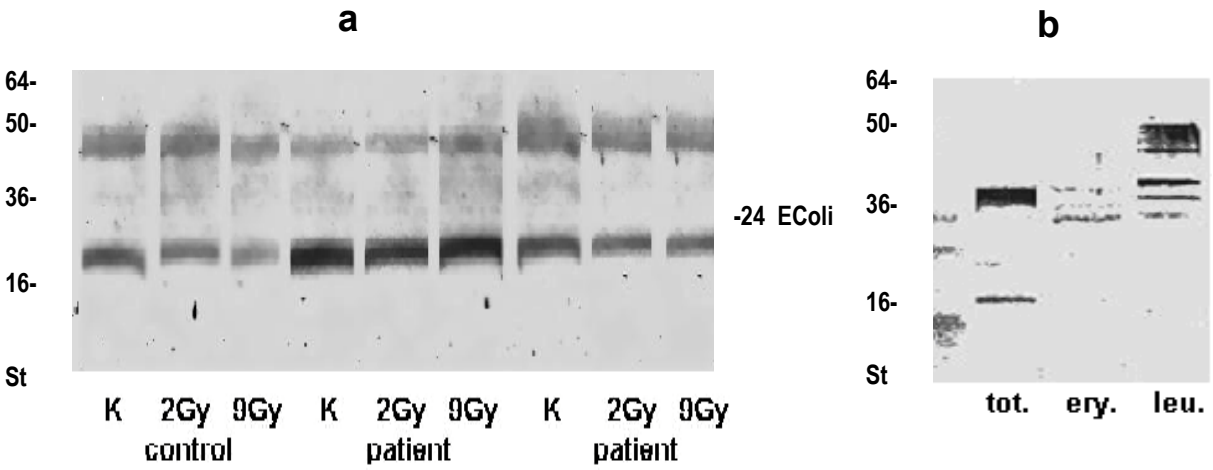

Figure 1. Western blot profiles of Mn SOD in blood cell extracts. *a) BC patients and respective control extracts irradiated with 0, 2- or 9 Gy of ^{60}Co-gamma rays; b) total blood cells (tot), erytrocytes (ery) or leukocytes (leu) of control sample. The positions of molar mass standards (Mm x 10^3 g/mol), and of the purified swine Mn SOD (E.coli, Mm = 24 x 10^3 g/mol) are indicated by dashes.*

Further analysis of Mn SOD levels was performed in the blood cell extracts obtained from the two groups of patients with BC: age group 30-45 (Figure 2c: premenopausal, n = 7, clinical stage 1- 3), and age group 46-60 (Figure 2d: peri- and post-menopausal women, n=14, clinical stage 3 or 4). The respective healthy women groups were used as controls: age group 30 – 45 (Figure 2a: premenopausal n=13) and age group 46-60 (Figure 2b: peri- and post-menopausal women, n=12). The radiation challenge with 2Gy and 9Gy of gamma-rays from ^{60}Co radiation source, was also carried out in all groups, and the levels of MnSOD were determined by the dot blot analysis. The intensity of immunostaining in each sample was quantified by the laser the densitometry and expressed in arbitrary units (AmU, mean ± S.E.M.) (Figure 2 a-d). The results indicated that most of 24 control samples had initial intracellular MnSOD levels below 50 AmU. After irradiation the control MnSOD levels were changed differently in each individual *i.e.* the enzyme levels increased in some controls, while they decreased in the others. The increase was more frequent in younger controls at 2Gy, while the decrease was more frequent in the older controls at 9 Gy. However, even after irradiation MnSOD levels mainly remained under or close to 50 AmU, irrespective of the radiation dose applied. In contrast to that, in 5 out of 19 BC patients initial MnSOD levels were above 50 AmU, and 11 out of 19 patients responded to irradiation with the significant increase in MnSOD level, which, in 6 cases was

even above 100 AmU. The pattern of MnSOD level after irradiation showed individual differences, but the equal number of BC patients has responded with the increase in the enzyme level, as with the decrease. This was found irrespective of the radiation dose applied. However, the extent of radiation-induced MnSOD increase was much higher than the extent of enzyme decrease. Thus, post irradiation with 2 Gy MnSOD level was over 150 AmU in one BC patient, while post 9 Gy it was 150-200 AmU in four BC patients.

When the average group values of intracellular MnSOD levels were analysed (Table 1a,b) they were 24.4 ± 9.6 AmU (mean ± S.E.M., n=12) in the younger control group (Table 1a, age 30-45), and 30.7 ± 15.0 AmU in the older control group (Table 1a, age 45-60, n=12). After irradiation with 2- and 9 Gy of ^{60}Co gamma-rays, a slight increase in the average value was found in the younger group of controls (32.4± 14.5 AmU, and 31.2 ± 21.3 AmU, respectively), while a slight decrease was found in the older group of controls (27.2 ± 13.1 AmU, and 29.3 ± 15.7 AmU, respectively). However, these differences were statistically insignificant when analysed by Multy factorial ANOVA.

When the average group values of intracellular MnSOD levels were analysed in the two age groups of BC patients, they were 45.6 ± 11.5 AmU and 46.6 ± 27.6 AmU, for the younger *vs.* older group, respectively (Table 1c,d). After irradiation the average group values of intracellular MnSOD levels were increased in both BC patients groups. They were 57.1 ± 41.4 AmU, and 56.8 ± 47.5 AmU after 2 Gy irradiation in the younger vs. older BC patients group. After 9 Gy irradiation the MnSOD levels were 84.7 ± 56.9 AmU, and 52.8 ± 46.0 AmU, respectively (Table 1c,d).

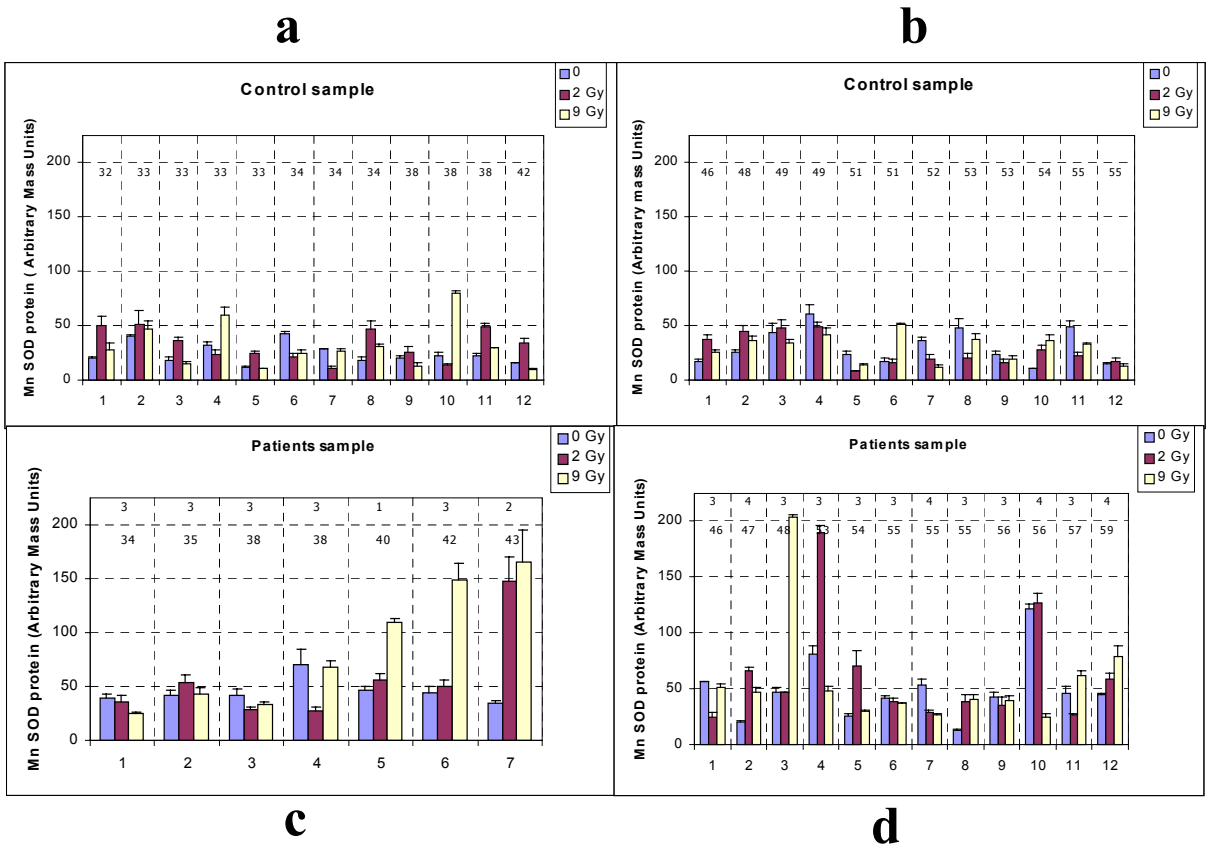

Figure 2. Mn SOD levels in untreated and irradiated blood cell extracts of patients with BC: a, b *- control samples;* ***c,d-*** *BC patients; the age and the BC disease stages are indicated above each sample. Mn SOD protein levels are expressed in arbitraty mass units (AmU). The error bars indicate mean ± S.E.M of the three separate measurements.*

Sample	Mn SOD in control samples (AmU)				Mn SOD in BC samples (AmU)			
	a		b		c		d	
	age group 30-45 n=12		age group 46-60 n=12		age group 30-45 n=7		age group 46-60 n=12	
	interval	*mean±SD*	*interval*	*mean±SD*	*interval*	*mean±SD*	*interval*	*mean±SD*
0	*11.4 – 42.5*	**24.4** ± 9.6	*16.8 – 60.8*	**30.7** ± 15.0	*34.5 – 70.2*	**45.6** ± 11.5	*20.6 – 121.4*	**46.6** ± 27.6
2Gy	*10.7 – 63.0*	**32.4** ± 14.5	*8.3 – 49.1*	**27.2** ± 13.1	*27.8 – 147.1*	**57.1** ± 41.4	*24.5 – 190.0*	**56.8** ± 47.5
9Gy	*9.9 – 80.0*	**31.2** ± 21.3	*12.2 – 51.2*	**29.3** ± 15.7	*25.3 – 165.9*	**84.7** ± 56.9	*24.7 – 203.6*	**52.8** ± 46.0

Table 1. Mn SOD levels in the control and BC blood cells: *the characteristic Mn SOD level intervals, the mean values and the standard deviations for each age group is given before and after irradiation with 2- and 9 Gy of ^{60}Co gamma-rays.*

If the mean values of BC patients groups were compared with the mean values of respective controle groups, the following observations could be made: firstly, the initial MnSOD levels were higher in BC patients; secondly, the standard deviations which illustrate the interval of MnSOD values characteristic for each group, were much broader for irradiated BC patients than for controls; and thirdly, while younger controls responded to irradiation with MnSOD increase, and older controls with the dicrease, in all BC patients MnSOD level was increased after irradiation.
The statistic Multy factorial ANOVA analysis confirmed the significant changes in the MnSOD protein levels betweein helthy controle samples and patients with BC ($F=25.166$, $p<0.001$). It also indicated that neither the age *i.e* menstrual status of BC patients and controls, nor the radiation dose used in blood cell challange test, showed any statistically significant effect.

4. Discussion

Radiotherapy has a very important role in treatment of breast cancer (BC) tumours, but it's practice is limited due to potentially harmful demages of the surounding healthy tissues. The ionizing radiations increase the concentration of reactive oxygen species (ROS), which cause massive oxidative damage of tissue DNA, proteins and lipids [4]. The helthy tissue damage may be decreased by protective low molar mass antioxidants, or higher molar mass antioxidants, such as various antioxidant enzymes, especially various superoxide dismutases [9]. To accomodate radiotherapeutical tratment for each individual patient, it my be very usefully to determine antioxidant repair capacity of exposed healthy tissue to radiation illustrated by the level of superoxide dismutases.

The major aim of this study was to acquire the primary information on individual variations in Mn superoxide dismutase (Mn SOD) protein levels which were assayed by quantitative immunoblotting and laser scanning densitometry. MnSOD was studied in the blood cell extracts obtained from 19 BC patients. According to their hormonal status the BC patients were devided in two age groups:

premenopausal group (age 30-45) and peri/post-menopausal women group (age 46-60). The BC patients of both groups were mostly diagnosed with grade 3 and 4 of the disease (Figure 2). Two respective groups of healthy women, age 30-45 (n=12), and 46-60 (n=12) were also analysed for Mn SOD blood cells level. Based on the notion that ionizing radiation is acting through increase of ROS *i.e.* through increase of $O_2^{\bullet -}$ it was expected that *in vitro* challenge with 2 Gy and 9 Gy of ^{60}Co gamma-rays [10] would result in Mn SOD changes reflecting the individual radiation response.
As documented in this preliminary study, the initial levels of blood cell MnSOD were higher in BC patients taken either individually or as a group mean values. The difference between healthy women and BC patients in the young group was *cca* 20%, while it was *cca* 16% in the older groups. I*n vitro* radiation challange either with 2Gy or 9Gy did not lead to statistically significant changes when either patients or controls were observed as a group. However, marked individual increase in MnSOD was observed in several patients, and in several controls. The increased MnSOD protein in irradiated BC patient samples were observed in the younger group especially at the dose of 9Gy (2-3 fold in respect to their own initial levels), when MnSOD values were as high as 150-200 AmU. The increase in some of the irradiate controls was also 2-3 fold, but their absolute values were still much lower *i.e.* around 50 AmU.

In spite of still existing controversy on the use of MnSOD as a biomarker for prediction of radiotherapy effects in some tumors [11], literature data agree that MnSOD increase induced either by irradiation or by a genetic manipulations plays an important part in radiation resistance of both tumor and healthy tissues [11–13].
Previous studies also showed increased production of superoxide anion, and increased activity of SODs in BC patients [14].

The *in vitro* radiation challenge test of MnSOD expression showed that while healthy controls did not show marked enzyme induction, some of the BC patients had markedly increased enzyme level post treatment. Thus, even if the averrage groups values did not chenge significantly, for some BC patients *in vitro* radiation challenge test of MnSOD expression seemed to be a good screening test selecting them further for preclinical tests of resistence to radiation-induced cell killing or for clinical follow up of therapy linked fibrosis.
Our study also indicated that the level of MnSOD protein was constantly higher in blood cells of BC patients, irrespective of their age *i.e.* their menstrual status, or the clinical stage of their disease. In considration to that, it is concluded that MnSOD could also be a relevant biomarker for the presence of BC, *i.e.* for the system reaction to the presence of malignant tumour.
Zhongkui Li *et al.* reported that the GADD153 (a protein involved in the repair of DNA double strand breaks) is specifically regulated by MnSOD, as transcription of this gene was 33 fold increased in MnSOD expressing tissues [15]. Bearing in mind that superoxide anions are involved in DNA damage, it may be speculated that in addition to MnSOD, GADD153 may represent potential biomarker illustrating individual sensitivity of either normal individuals or BC patients to oxidative stress inducing agents, such as ionizing radiation. Consequently, GADD153 would also be included in our further studies.

5. Conclusion

The *in vitro* radiation challenge test of blood cell MnSOD level indicate that the enzyme expression may be a good screening test for selection of BC patients that may express increased resistance to oxidative stress . The initial MnSOD levels in healthy individuals *vs.* BC patients also indicate that the enzyme expression may be a relevant biomarker for the presence of BC *i.e.* for the system reaction to the presence of malignant tumour.

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