

Superoxide dismutases and p53 protein levels in blood cells of breast cancer patients

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Summary

Purpose: Radiation treatment of breast cancer (BC) often results in post-therapy complications. The undesired sequelae could be avoided by the diagnostic screening of biomarkers for prediction of ionizing radiation (IR)-linked injury of healthy tissues.

Patients and methods: The expression of antioxidative defence enzymes CuZn- and Mn-superoxide dismutase (CuZnSOD, MnSOD) and tumor suppressor protein p53 was measured in blood cells of 19 women with BC (age groups 30-45 and 46-60 years) and respective controls. The proteins were detected by specific immunostaining and quantified by laser-scanning densitometry.

Results: Constitutive expression of CuZnSOD was significantly elevated in the group of BC patients (up to 254 arbitrary units, AU/mL) relative to the control group

(105-130 AU/mL). The constitutive expression of MnSOD was elevated (up to 94 AU/mL) in the group of BC patients relative to the controls (53-56 AU/mL). p53 was also constitutively more expressed in BC patients (35-42 AU/mL) than in controls (32-33 AU/mL). Both MnSOD and p53 were inducible by ⁶⁰Co gamma-ray IR (up to 170 AU/mL and 51 AU/mL, respectively) in the BC patient group. The levels of IR-induced p53 correlated inversely with MnSOD levels.

Conclusion: The constitutive expression of all 3 proteins could be a useful biomarker for the presence of BC, but only MnSOD overexpression may be the predictive biomarker for selection of BC patients that would be less susceptible to IR-linked complications.

Key words: blood cells, breast cancer, ionizing radiation, p53, superoxide dismutase

Introduction

One of the major problems in IR therapy of BC is the relatively frequent occurrence of undesired side-effects that limits its efficacy [1,2]. The ability to predict and alleviate these side-effects would greatly enhance the quality of life of cancer patients after IR

treatment. The normal tissues that are mostly affected by IR are lymphoid and hematopoietic organs, as well as different epithelia [3]. The cytotoxic effects of IR are partly due to the increase in concentration of reactive oxygen species (ROS), which cause massive oxidative damage of DNA, proteins and lipids [4,5]. To protect against these effects, cells have evolved both non-enzymatic and enzymatic antioxidant defence systems, localized in varying cells compartments [6]. The first line in enzymatic defence in the cell cytosol compartment is CuZn-superoxide dismutase (SOD1, EC 1.15.1.1, dimer molar mass Mm=32,000 g/mol), while its mitochondrial counterpart is Mn-superoxide dismutase (SOD2, EC 1.15.1.1, tetramere Mm=88,000 g/mol) [7]. Both enzymes are highly efficient in scavenging superoxide (O₂^{•-}) radical by catalysing its dismutation into hydrogen peroxide (H₂O₂) and molecular oxygen (O₂) [6]. The overexpression of MnSOD both at the mRNA and protein level is cor-

Received 14-07-2004; Accepted 02-08-2004

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related with increased radiation resistance [8], while the deficiency or low level enzyme activity of MnSOD are associated with increased radiosensitivity and cell death induction [9]. It is well documented that the healthy tissue damage may be decreased by local or systematic application of SODs [1,2,10]. Another protein involved in cellular response to IR-induced oxidative stress is the nuclear DNA-binding transcription factor p53, which regulates several systems of cellular defence against DNA damage [11]. Dependent on the extent of cell damage p53 activates genes that control cell cycle (Waf-1/p21), and DNA repair (Gadd45), or genes executing cell suicidal program [12]. At least in part, the action of p53 is accomplished through downregulation of MnSOD expression resulting in ROS accumulation and initiation of the cell suicidal program [13]. p53 is active as a tetramer, thus its activity is highly dependent on its concentration in the cell. In the normal unstressed cells p53 is a short-lived protein, which is maintained at low, often undetectable levels, via its interaction with mouse double minute 2 (MDM2), and ubiquitin ligase [14]. The p53 activity is stabilized by protein phosphorylation, which leads to the release of MDM2, and inhibition of p53 nuclear export [14]. The level of stabilized p53 is much higher in radiosensitive tissues than in radioresistant ones [15]. It was observed that mitochondrial MnSOD levels are in inverse correlation with p53 function, thus the enzyme is being expressed at considerable levels in cells in which p53 is either mutated or virtually absent, but barely detectable in the cells expressing high amounts of wild-type p53 [16]. High levels of MnSOD downregulate p53 gene expression, which could also play a role in the negative modulation of the p53 functions [16]. Thus, at least in part, the interplay between cellular p53 and MnSOD could predict both the efficacy of tumor cell killing and the probability of healthy tissue complications. Some reports even indicate that temporary inhibition of p53 might be useful for reducing the side-effects of cancer therapy [17].

Based on the presented data it is speculated that both constitutive and IR-induced expression of SODs and p53 in healthy tissues of BC patients may be of help for the prediction of their response to curative IR-therapy, as well as for adjustment of supplementary SOD treatment in cases of radiation-induced fibrosis [1,2]. In the present study the peripheral blood cells, CuZnSOD, MnSOD and p53 were measured in BC patients and the respective controls, with the aim to determine the differences in constitutive and ^{60}Co gamma-ray IR-induced expression of the proteins.

Patients and methods

The levels of CuZnSOD, MnSOD and p53 were determined in peripheral blood cells of 19 patients with BC at diagnosis, prior to any clinical treatment. The assay was performed in 2 groups of women with BC: age group 30-45 years (premenopausal, n=7, clinical stage 1-3), and age group 46-60 years (peri- and post-menopausal women, n=12, clinical stage 3 and 4). Twenty-four healthy women [age group 30-45 years (n=12) and age group 46-60 years (n=12)] were used as controls. Both patients and controls gave informed consent. The study was approved by the Institution Review Board at the Institute of Oncology and Radiology of Serbia.

Aliquots of heparinized venous blood were cultured in 10% RPMI after 0-, 2- and 9- Gy irradiation on ^{60}Co gamma-ray source. The dose rate was 20 Gy/h. After centrifugation at 1,500 rpm for 5 min at room temperature the cell pellets were lysed by adding lysing buffer containing 0.32 M sucrose, 10 mM TrisHCl, 5 mM MgCl₂, and 1% Triton-100. The protein concentration in the cell lysate was determined by the Lowry method [18]. Aliquots of blood cell extracts were mixed with denaturing buffer according to Laemmli [19], boiled (100° C, 2 min), cooled and applied to nitrocellulose membrane using Manifold device (Schleicher and Schuell, Inc., Keene, NH, USA). Unbound sites on the membrane were blocked by 10 mM Tris buffer pH 7.4 supplemented with 150 mM NaCl, 1% BSA and 0.1% Tween-20 (TBST buffer). The membrane was incubated overnight (4° C) either with polyclonal Rabbit Anti-Human CuZn- or MnSOD antibodies (SOD-100 or SOD-101, 0.2 mg/mL TBST; Stressgen Biotechnologies, Victoria, BC, Canada). The secondary Goat Anti-Rabbit IgG Alkaline Phosphatase Conjugate SAB-301 (Stressgen Biotechnologies, Victoria, BC, Canada) was used for the detection. p53 was quantified using monoclonal Anti-Human p53 antibodies (Ab-3, 10 mg/mL TBST; Oncogene Research Products, Cambridge, MA, USA) and Goat Anti-Mouse IgG Alkaline Phosphatase Conjugate (Novagen Madison, WI, USA). The quantification of specific antigen bands was achieved by UltraScan XL scanning laser densitometry and PC processing. The level of proteins was expressed in AU per cells obtained from 1 mL of blood, from 4-6 independent measurements. The experimental error of the measurements was \approx 15%. Multifactorial ANOVA (MANOVA) analysis was applied to estimate statistically relevant differences in respect to age, menopausal status, health conditions, and response to IR. If statistical significance was found, the Tukey post-hoc analysis was performed. Significance was accepted for $p < 0.05$.

Results

Results presented in Table 1 show intervals of individual expression of the proteins and group mean

values with standard deviation. The group mean values with statistics are presented in Figure 1. It was observed that the controls had markedly lower constitutive levels of both CuZnSOD and MnSOD, and

Table 1. Blood cell levels of CuZnSOD, MnSOD and p53

Expression	Controls (AU/mL)				BC patients (AU/mL)			
	age 30-45		age 46-60		age 30-45		age 46-60	
	Interval	Mean±SD	Interval	Mean±SD	Interval	Mean±SD	Interval	Mean±SD
<i>CuZnSOD</i>								
Const.	76.2 – 182.6	129.8±26.8	66.8 – 152.6	105.4±25.0	160.2 – 292.6	214.8±44.2	167.8 – 331.8	253.6±52.8
<i>MnSOD</i>								
Const.	22.7 – 121.5	55.8±27.7	20.8 – 97.1	53.4±26.1	69.0 – 140.4	91.2± 23.0	41.2 – 242.8	93.2±55.2
2 Gy	21.4 – 126.0	64.8±29.0	16.6 – 98.2	54.4±26.2	55.7 – 294.2	114.1± 82.7	49.0 – 380.0	124.9±98.1
9 Gy	19.8 – 160.0	64.4±42.6	24.4 – 102.4	58.6±31.4	50.6 – 331.8	169.4±113.8	49.4 – 407.2	105.6±92.0
<i>p53</i>								
Const.	25.8 – 42.8	32.4±4.8	12.8 – 54.8	33.2± 9.2	23.8 – 58.4	41.8±12.2	21.8 – 47.8	35.8± 8.6
2 Gy	18.8 – 50.8	31.0±7.8	24.4 – 44.2	35.0± 6.2	26.2 – 83.8	47.6±19.6	33.4 – 84.2	50.4±15.2
9 Gy	28.6 – 46.8	35.2±5.8	21.6 – 57.2	38.4±10.0	21.8 – 65.8	43.6±14.0	16.6 – 76.4	38.6±13.8

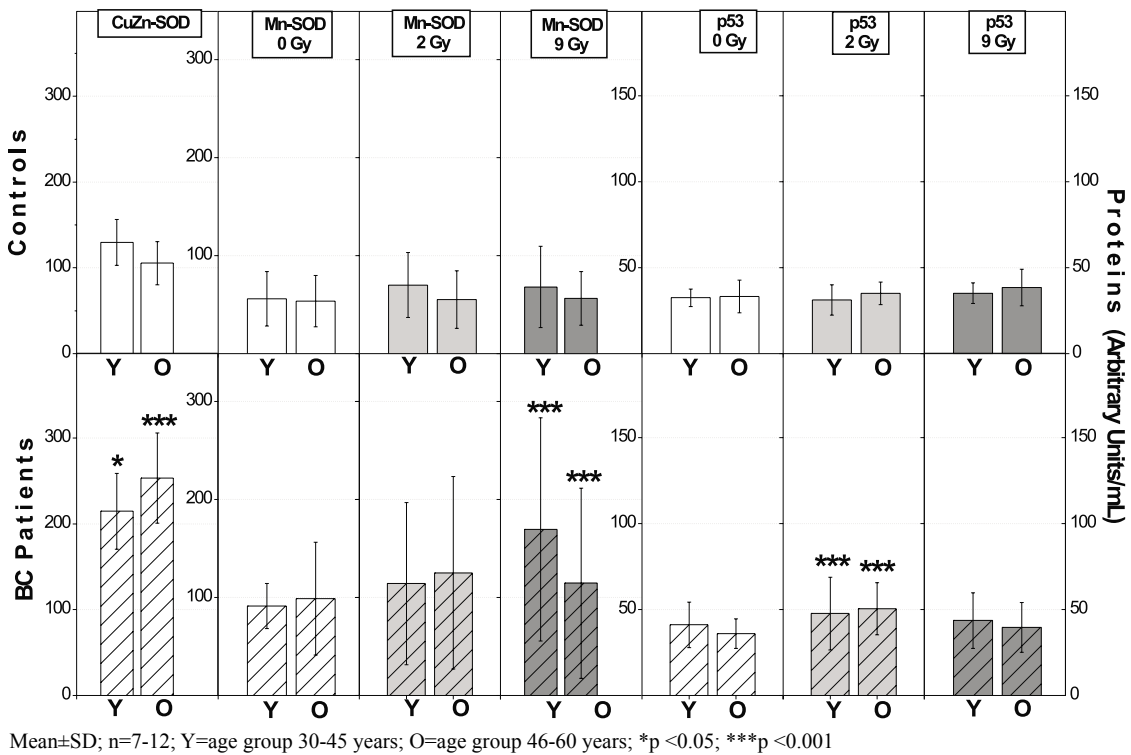


Figure 1. Blood cell levels of CuZnSOD, MnSOD and p53.

slightly lower constitutive level of p53 compared to BC patients (Figure 1). This was valid for all control samples irrespective of the age group (premenopausal *versus* peri/post menopausal).

The individual constitutive levels of CuZnSOD in controls were in the range of 67-183 AU/mL with a slight tendency to decrease with advancing age (Table 1). The patients with BC had much higher individual constitutive expression of CuZnSOD and much more pronounced individual differences, ranging from 160-332 AU/mL, somewhat more pronounced with age and disease progression.

The results of MnSOD immunostaining indicated that constitutive levels of the enzyme in controls were in the range of 21-122 AU/mL (Table 1). The patients with BC had markedly higher individual constitutive expression of MnSOD ranging from 41-243 AU/mL. After irradiation with 2- or 9 Gy, the younger controls responded with enzyme induction up to 160 AU/mL, while the older controls were virtually unaffected. After irradiation younger BC patients responded with MnSOD increase up to 332 AU/mL, while in older patients MnSOD was induced up to 407 AU/mL.

The results of p53 immunostaining indicated that constitutive levels of the protein in the control groups were in the range of 13-55 AU/mL (Table 1). In BC patients the constitutive p53 levels ranged between 22-59 AU/mL. After irradiation with 2- or 9 Gy, the younger controls responded with p53 induction up to 51 AU/mL, while in the older controls p53 increased up to 57 AU/mL. After irradiation both younger and older BC patients responded with p53 increase up to 84 AU/mL.

Taken together, in both groups of BC patients constitutive CuZnSOD and MnSOD levels were markedly higher (65-140% and 75-89%, respectively), while p53 levels were slightly higher (10-29%) relative to the respective controls. MANOVA analysis, followed by the Tukey post hoc test, showed differences between BC patients and healthy blood donors which were significant for CuZnSOD ($p < 0.001$, Figure 1). The inter- or intra-group differences for either control or BC patient proteins were not significant when age, hormonal (menopausal) status or disease stage were taken in consideration. Significant IR induction of MnSOD in BC samples was found at 9 Gy ($p < 0.001$, Figure 1), and significant IR induction of p53 was found at 2 Gy ($p < 0.001$, Figure 1).

Discussion

The reasons for the increased constitutive levels of CuZnSOD and MnSOD expression in BC patients

could be multiple. For example, it was claimed that malignant tumor, as a tissue with high proliferative index, represents a high oxidative burden to the organism [20], and the presence of BC may induce expression of SODs throughout the organism, primarily in circulating cells [21]. In fact, increased ROS production in the plasma of BC patients correlating with the clinical disease stage was previously observed [20]. In addition, as it was found that the activity of SODs is significantly lowered in BC [22], the increased expression and activity of the enzyme in the circulation may perhaps compensate for the decreased antioxidant capacity of the tumor tissue [21]. The marked IR-induction of MnSOD observed in our study is not unexpected, as IR increases ROS, so that the enzyme increase may represent a defence mechanism leading to increased resistance to oxidative stress. Also, the observation that the initial increase in p53 at 2 Gy is followed by its decrease at 9 Gy, which correlates with the highest level of MnSOD expression, may be explained by the mutual negative regulation of p53 and SOD2 gene expression [13,16]. In fact, the finding of high blood cell p53 concomitant with the low MnSOD may indicate increased individual susceptibility to IR-linked injury and healthy tissue cell death, while low blood cell p53 and high MnSOD may be potentially linked with increased individual resistance to the IR-induced oxidative stress.

Based on the presented data it may be concluded that the constitutive expression of all 3 proteins could be a useful biomarker for the presence of BC, i.e. for the systemic reaction to the growing breast tumor. However, only MnSOD overexpression linked to p53 downregulation may be the predictive biomarker for selection of BC patients that would be less susceptible to IR-linked complications.

Acknowledgement

The presented study was supported by the Ministry of Sciences and Environment Protection of the Republic of Serbia, Grant BOI1953.

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