ANTIOXIDANT STATUS IN BREAST CANCER PATIENTS OF DIFFERENT AGES AFTER RADIOTHERAPY

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Abstract — In this study we investigated the effects of breast cancer radiotherapy on the antioxidant (AO) enzyme activities of copper, zinc superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as on the concentration of reduced glutathione (GSH) and lipid peroxides (LP) in blood of patients aged 45-58 years and older than 60 years. The results show that in blood of patients aged 45-58 years, radiotherapy increased the activities of CuZnSOD, CAT, and GR, as well as the concentration of GSH, without affecting the activity of GPx and concentration of LP. In patients older than 60 years, radiotherapy increased the activities of CuZnSOD and CAT, lowered the activity of GPx and concentration of GSH, and increased the concentration of LP. Our results indicate that the response to radiotherapy involves age-related impairment of AO capacity for elimination of H_2O_2, causing oxidative damage to blood cells. This suggests that cytotoxic effects of radiation on healthy tissues might be more pronounced during the aging of breast cancer patients, and should be considered in the further development of individualization protocols in cancer radiotherapy.

Key words: Antioxidant enzymes, lipid peroxides, breast cancer

INTRODUCTION

Radiotherapy-induced increased generation of reactive oxygen species (ROS) exceeding the protective capacity of the antioxidant and DNA repair system causes death of cancer cells, but also has toxic side effects on healthy tissues (Nair et al., 2001). Moreover, differences in the activities of AO enzymes may be associated with the complex phenomena of radiosensitivity and radioresistance. Increase of both malignancy and radiosensitivity were shown to be associated with decrease of MnSOD and GPx activities, as well as GSH content, in rat epithelial cell lines (Bravard et al., 2002). In a radioresistant variant clone from a human glioblastoma cell line, the activities of SOD, CAT, GPx, and GR were found to be activated up to five-fold when compared to the parent cells after irradiation (Lee et al., 2004). In addition, severe side effects commonly arising from radiotherapy often prevent patients from completing the treatment course and largely determine the quality of life following radiotherapy. Estimation of oxidative damage and the AO defense capacity of healthy tissues in the response to radiotherapy can therefore contribute to the development of successful radioprotection strategies. Our previous research showed that the activity of MnSOD is inversely correlated with the yield of γ-radiation–induced micronuclei *in vitro* in human blood lymphocytes, suggesting a base for a rapid predictive assay of radiosensitivity in a clinical setting (Pajović et al., 2000). Our results also showed that activity of SOD, particularly MnSOD, significantly contributes to the relative biological effectiveness of proton irradiation *in vitro* as measured by the dose-dependent production of dicentrics and micronuclei in human lymphocytes (Joksić et al., 2000).

By initiating autocatalytic lipid peroxidation, ROS generate a large variety of potential toxic

products (such as LP) that are often used as markers of lipid peroxidation and oxidative stress in human pathology and toxicology (Mayne, 2003). Superoxide dismutase catalyzes dismutation of the superoxide radical ($O_2^{\bullet-}$) into H_2O_2, which is further decomposed by the action of CAT and GPx (Ambrosone, 2000). In conjunction with GPx, GSH detoxifies peroxides and a wide range of xenobiotics and carcinogens. Restoration of reduced GSH is made possible by GR (Abou Ghalia and Fouad, 2000).

Cancer is among the degenerative diseases associated with aging, which also include cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. The functional degeneration of somatic cells during aging appears, in good part, to contribute to these diseases. Although ROS (oxidative stress) are recognized to play a significant role in breast carcinogenesis (Ambrosone, 2000; Kang, 2002), as well as in the aging process (Droge, 2003), we found no published data regarding age-related modulation of oxidative stress and the AO system in breast cancer radiotherapy. In this work, we therefore investigated the effects of radiotherapy on activities of AO enzymes (CuZnSOD, CAT, GPx, and GR), GSH concentration in blood cells, and LP concentration in blood plasma in breast cancer patients of different ages.

MATERIALS AND METHODS

The study included breast cancer patients from the Institute of Oncology and Radiology of Serbia, Belgrade, divided according to their age into two age groups, 45-58 years (n=7, median age 51.3) and above 60 years (n=9, median age 69.4). All of them were newly diagnosed and clinically classified as patients in stages IIIA (n=11) and IIIB (n=5) according to the system of the International Union Against Cancer (UICC) (1997). Breast cancer cases were further classified as T2N2M0 (n=9), T3N2M0 (n=2), and T4bN1M0 (n=5), and tumors were histologically diagnosed as invasive ductal (n=8), invasive lobular (n=6), and medullary (n=2) carcinomas. All patients in the age group of 45-58 years were peri-menopausal, whereas patients older than 60 years were post-menopausal. Patient characteristics according to their age are presented in Table 1. Patients were submitted to radiotherapy with a total dose of 45-51 Gy and fraction size of 3 Gy for breast tumors, and with a total dose of 45 Gy and fraction size of 3 Gy for regional lymphatics, over a period of 6 weeks (Linear Accelerator, 6 MeV). In accordance with the ethical guidelines of the Helsinki Declaration, informed consent was obtained from all participants.

Heparinized venous blood specimens were obtained on the day of the beginning of the radiotherapy regimen and 24 h after its completion. Blood specimens were centrifuged at 2500g for 5 min at 4°C (Eppendorf centrifuge 5417, Eppendorf AG, Hamburg, Germany). Plasma samples were collected, kept at -70⁰C, and after defrosting centrifuged at 11000g for 1 min at 4°C. Clarified plasma preparations were used for LPO assay. Blood cells from pellets were washed two times in cold 0.9% NaCl and lyzed in 2 volumes of ice-cold demineralized ultrapure water (MilliQ reagent grade water system, Millipore Corp., Bedford, MA, USA), after which the crude lyzate was kept frozen at -70°C before being used for blood cell extracts and clarified lyzate preparations. The crude lyzate was used for CAT and GPx assays and protein concentration measurements. Hemoglobin was removed from the crude lyzate by adding chloroform and ethanol (the lyzate: chloroform: ethanol volumetric ratio was 1: 1: 0.6). After centrifugation at 3000g for 10 min at 4°C (Eppendorf centrifuge 5417, Eppendorf AG, Hamburg, Germany), the upper aqueous layer was collected and used for CuZnSOD assay. Following blood cell stroma removal from the crude lyzate by centrifugation at 8600g for 10 min at 4°C, the clarified lyzate was used for GR assay. After protein precipitation from the crude lyzate (the lyzate: precipitation reagent volumetric ratio was 1: 3) and centrifugation at 10000g for 5 min at room temperature, the supernatant was used for GSH assay.

Assays for CuZnSOD, GPx, GR, GSH, and LPO were performed using methods and reagents from Bioxytech^R Assays (OXIS International Inc., Portland, OR, USA). The unit of SOD activity was defined as the amount of enzyme that doubles the autooxidation rate of the control blank under the assay condi-

Table 1. Breast cancer patient characteristics.

Age group	45-58 years (n=7)	above 60 years (n=9)
Median age (years, range)	51 (48-58)	69 (62-78)
Clinical stage		
Stage IIIA T2N2M0	4	5
T3N2M0	1	1
Stage IIIB T4bN1M0	2	3
Histological type		
Invasive ductal	3	5
Invasive lobular	2	4
Medullary	2	0
Menopausal status		
Peri-menopausal	7	0
Post-menopausal	0	9

tions. The unit of GPx activity and GR activity was defined as 1 μmol of NADPH oxidized per minute under the assay conditions. Determination of CAT activity was performed by the method of Beutler (1982). The unit of CAT activity was defined as 1 μmol of H_2O_2 decomposed per minute under the method conditions. The enzyme assays and concentration measurements were monitored spectrophotometrically (Perkin Elmer spectrophotometer, λ25, Perkin Elmer Instruments, Norwalk, CT, USA). Enzyme activities were expressed in U or mU per milligram of total cell protein (U or mU/mg protein). Concentrations of GSH and LPO were expressed in nmol/mg protein and pmol/mg of protein, respectively. Determination of protein concentration was performed by the method of Lowry et al. (1951) and expressed in mg/ml.

Statistical analyses were performed using the Origin 7.0 statistical package. Data were compared by the Student's paired t-test at a statistical significance level of $p<0.05$ and expressed as means ± SEM.

RESULTS

The results indicate that the AO status in patients' blood 24 h after the breast cancer radiotherapy regimen was completed differed significantly from the values before radiotherapy (Fig. 1). In patients aged 45-58 years, radiotherapy increased the activities of CuZnSOD (2.48±0.39 vs. 2.73±0.34 U/mg of prot.,

$p<0.05$), CAT (95.73±9.03 vs. 112.97±8.49 U/mg of prot., $p<0.05$), and GR (3.66±0.32 vs. 3.94±0.36 mU/mg of prot., $p<0.05$), as well as concentration of GSH (5.67±0.58 vs. 6.83±0.52 nmol/mg of prot., $p<0.05$). In this age group, GPx activity remained unchanged after radiotherapy (17.14±0.47 vs. 17.26±1.0 mU/mg of prot., $p>0.05$), and the concentration of LP was also steady (72.20±5.58 vs. 74.19±9.57 pmol/mg of prot., $p>0.05$).

As in younger ones, radiotherapy in patients older than 60 years increased the activities of CuZnSOD (3.09±0.21 vs. 3.48±0.22 U/mg of prot., $p<0.05$) and CAT (95.04±6.47 vs. 107.74±5.29 U/mg of prot., $p<0.05$), but simultaneously decreased the activity of GPx (17.46±0.50 vs. 14.65±0.93 mU/mg of prot., $p<0.05$) and the concentration of GSH (6.04±0.62 vs. 5.04±0.51 nmol/mg of prot., $p<0.05$). In this age group, radiotherapy did not significantly affect the activity of GR (3.53±0.38 vs. 3.00±0.29 mU/mg of prot., $p>0.05$), whereas it increased the concentration of LP (70.66±5.61 vs. 80.32±3.69 pmol/mg of prot., $p<0.05$).

DISCUSSION

Our results showed that the AO response to radiotherapy in the blood of breast cancer patients was significantly different in the examined age groups. In the patients aged 45 to 58 years, oxidative stress was not detected after radiotherapy, to judge from
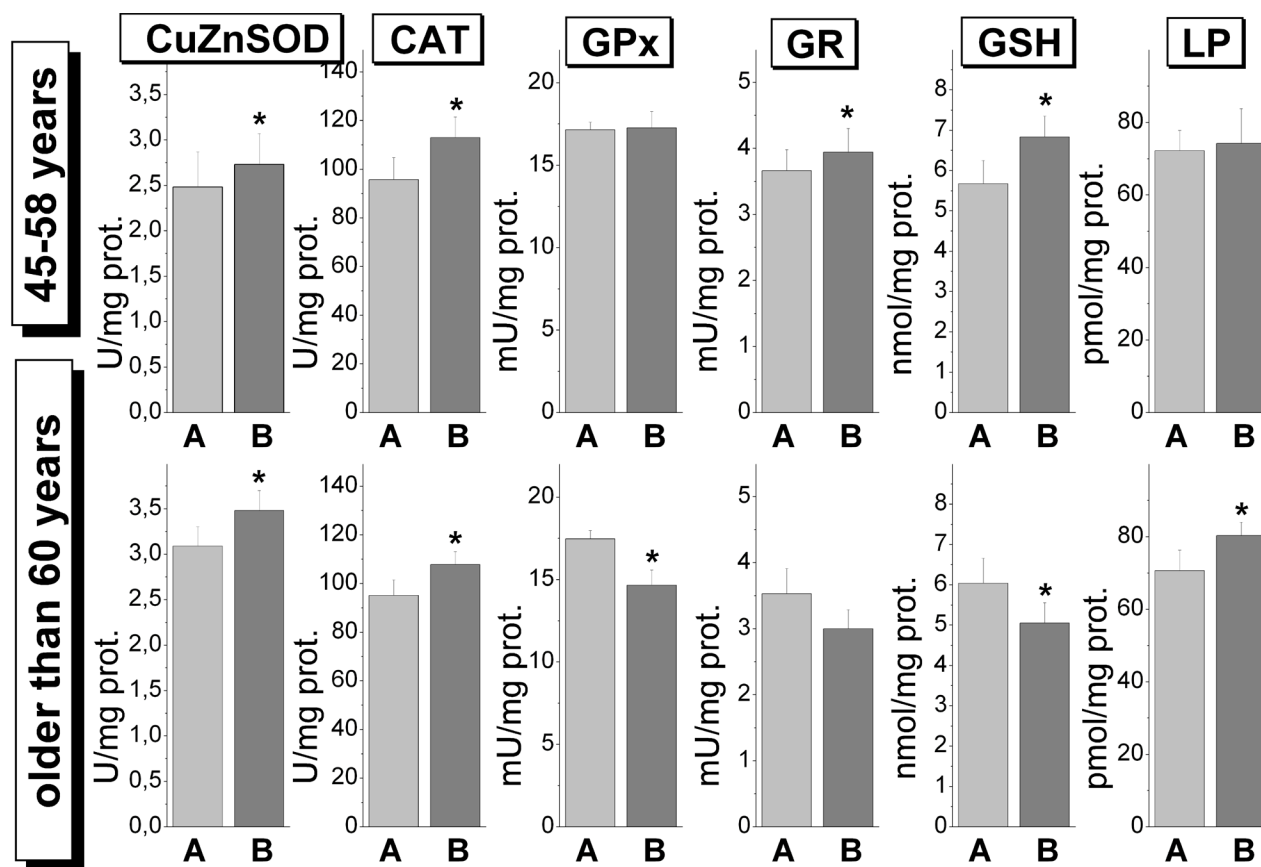
Fig. 1. Antioxidant enzyme activities and concentration of GSH and LP in the blood of breast cancer patients in the examined age groups before (A) and after (B) radiotherapy. The data are given in mean values ± SEM, * p<0.05 (Student's paired t-test).

the unchanged concentration of LP. At the same time, increased GR activity indicates increased production of reduced GSH, which is in accordance with its increased concentration in the blood. These results imply that increased activities of SOD and CAT, as well as increased concentration of reduced GSH, are sufficient for elimination of elevated $O_2^{\cdot -}$ and H_2O_2 production and suppression of oxidative stress induced by radiotherapy in the blood of younger patients.

In patients above the age of 60, radiotherapy induced occurrence of lipid oxidative damage. In this age group, the activities of CuZnSOD and CAT were increased after radiotherapy (as in younger patients), but the activity of GPx and GSH concentration were found to be significantly lowered. Based on this, it would appear that decreased capacity for

H_2O_2 elimination might be a cause of the detected oxidative damage. Hydrogen peroxide in increased concentrations, apart from causing direct oxidative damage, can generate extremely reactive $^{\cdot}OH$ radicals through interaction with transitional metals (Stohs and Bagchi, 1995). In addition to the radiotherapy-induced suppression of H_2O_2 detoxification reported in this work, human tumor cells were found to produce increased amounts of H_2O_2, keeping them under persistent oxidative stress (Szatrowski and Nathan, 1991). Breast cancer may also create systemic conditions that increase the level of H_2O_2 in circulating cells. Such systemic conditions may favor survival and further invasion of breast cancer cells (Adžić et al., 2006).

Free radicals and peroxides can be directly removed by the action of reduced GSH, which is

the main endogenous soluble antioxidant in mammalian cells (Balendiran et al., 2004). Reduced glutathione can act through formation of a disulfide mixture or by oxidation to GSSG, thus preventing damaging effects on tissues caused by peroxidation of membrane lipids (Toborek and Henning, 1994). A decreased level of GSH may cause increased oxidative damage to lipids, both through impairment of its direct antioxidative actions and through indirect influence *via* decreasing GPx activity where it is necessary as a co-substrate (Abou Ghalia and Fouad, 2000), both of which were recorded in patients above 60 years. Since the reduced activity of GR found in older patients, although not significant, coincides with a lowered concentration of GSH, it suggests a reduced level of GSH regeneration by GR in this age group.

Our results indicate that the response to radiotherapy includes age-related decrease of AO capacity for elimination of H_2O_2, thus causing the occurrence of oxidative damage to blood cells in progressive stages of breast carcinoma. This suggests that cytotoxic radiation effects on healthy tissues, induced by increased ROS production and causing oxidative damage to biomolecules, may significantly interfere with cell homeostasis in older patients with breast cancer as a consequence of the already present suppression of the AO system during the aging process. In a recently published work, we observed a deleterious contribution of the aging process to increase of oxidative stress, decrease of AO enzyme activities and concentration of GSH, and decrease of cytosol SOD enzyme expression in progressive stages of breast carcinomas. These results strongly emphasize the rising need for additional antioxidant support throughout the advancing aging process, particularly in middle-aged and elderly women with higher risk of breast carcinomas (Kasapović et al., 2008). To sum up, it may be important to consider circulating levels of antioxidants in predicting a woman's risk of breast cancer and individualizing breast cancer radiotherapy protocols (Pajović et al., 2000, 2003).

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АНТИОКСИДАТИВНИ СТАТУС КОД ПАЦИЈЕНТКИЊА РАЗЛИЧИТЕ СТАРОСТИ СА КАНЦЕРОМ ДОЈКЕ ПОСЛЕ РАДИОТЕРАПИЈЕ

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У овом раду испитивали смо ефекте радиотерапије канцера дојке на активност антиоксидативних (АО) ензима, бакар, цинк супероксид дисмутазе (CuZnSOD), каталазе (CAT), глутатион пероксидазе (GPx) и глутатион редуктазе (GR), као и на концентрацију редукованог глутатиона (GSH) и липидних пероксида (LP) у крви пацијенткиња старих од 45-58 година и старијих од 60 година. Резултати показују да у крви пацијенткиња старих 45-58 год., радиотерапија индукује повећање активности CuZnSOD, CAT и GR, као и концентрације GSH и не утиче на активност GPx и концентрацију LP. Код пацијенткиња старијих од 60 год., радиотерапија индукује повећање активности CuZnSOD и CAT, снижење активности GPx и концентрације GSH, као и повећање концентрације LP. Наши резултати указују да радиотерапијски одговор укључује снижење АО капацитета за уклањање H_2O_2 које је везано за старост, узрокујући оксидативна оштећења у ћелијама крви. Ово сугерише да цитотоксични ефекат радијације на здрава ткива може бити израженији током старења пацијенткиња са канцером дојке и требало би га узети у обзир приликом даљег развоја индивидуализације протокола у радиотерапији канцера.