Activities of Erythrocyte Antioxidant Enzymes in Cancer Patients

Clifford Abiaka,^{1*} Farida Al-Awadi,² Hilal Al-Sayer,³ Sima Gulshan,²
Abdulla Behbehani,³ and Medhat Farghally³

¹Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Kuwait University, Sulaibikhat, Kuwait

²Department of Biochemistry, Faculty of Medicine, Kuwait University, Safat, Kuwait

³Department of Surgery, Faculty of Medicine, Kuwait University, Safat, Kuwait

We measured the activities of erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GPX) before therapy in 97 patients with cancer in various sites (gastrointestinal tract (GIT) (n = 40), breast (n = 30), and others (n = 27)), and in 60 matched controls to assess antioxidant enzyme protection. Hemolysate hemoglobin (Hb) was measured spectrophotometrically. The activity of SOD (U/g Hb) was significantly lower in all sites (when all the cancer sites were considered as a group), GIT, breast, and other sites compared to the controls ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$ and $P < 0.0001$, respectively). The activity of GPX (U/g Hb) was signifi-

cantly decreased in all sites, GIT, and breast cancer sites than in the controls ($P = 0.024$, $P = 0.033$, and $P = 0.043$, respectively). Age showed a weak negative correlation with enzyme activities in controls and patients. There was no significant association between SOD and GPX activities in either the controls or the patients. These results suggest that there may be a greater antioxidant burden for SOD than GPX in cancer, and that a weak association exists between the activities of the two enzymes in antioxidant protection. *J. Clin. Lab. Anal.* 16:167–171, 2002. © 2002 Wiley-Liss, Inc.

Key words: superoxide dismutase; glutathione peroxidase; hemoglobin; breast cancer; gastrointestinal tract cancer; other cancer sites

INTRODUCTION

Antioxidant enzymes form part of the body's defense against free radical attack (1) and are derived from *in vivo* synthesis (2). The enzyme SOD (EC 1.15.1.1; $O_2^{\cdot-}:O_2^{\cdot-}$ oxidoreductase; SOD, m.w. 32,500) consists of a class of metallo-proteins, which includes a copper zinc-superoxide dismutase (CuZn-SOD) that is found abundantly in the cytosol of oxygen-metabolizing eukaryotic cells (3,4). SOD is highly efficient in scavenging superoxide ($O_2^{\cdot-}$) radical by catalyzing the disproportionation of the radical to hydrogen peroxide (H_2O_2) and molecular oxygen, thereby greatly accelerating the rate at which $O_2^{\cdot-}$ is removed (5).

Glutathione peroxidase (EC 1.11.1.9; glutathione: hydrogen peroxide oxidoreductase) is a selenoprotein with a molecular weight of 72,000–84,000 dalton that is located in the cytosol of most tissues. The enzyme catalyzes the decomposition of both hydrogen peroxide and organic peroxides (ROO) at the expense of reduced glutathione (GSH) with the formation of glutathione

disulphide (GSSG), water, and organic alcohol (ROH). The total activity of GPX (selenium- and non-selenium-dependent) is determined by the coupled enzyme procedure, with cumene hydroperoxide as oxidant (6). In erythrocytes from adult humans, all GPX activity appears to be selenium-dependent (7), and therefore the measurement represents selenium-GPX.

The aim of this study was to measure the activities of SOD and GPX in the erythrocytes of patients with cancer expressed in different anatomical sites to assess antioxidant enzyme protection.

*Correspondence to: Clifford Abiaka, Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Kuwait University, P.O. Box 31470, Sulaibikhat 90805, Kuwait. E-mail: clifford@hsc.kuniv.edu.kw; clifab@hotmail.com

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MATERIALS AND METHODS

Subjects

Ninety-seven cancer in-patients (37 males and 60 females; 6–85 years old) were studied. The cancers were grouped according to anatomical sites: gastrointestinal tract (GIT) ($n = 40$), breast ($n = 30$), and others ($n = 27$). The GIT sites included cancers expressed from the mouth to the rectum; “others” refers to cancers expressed in sites other than the breast and GIT. Twenty-five patients out of 97 were smokers, but there were no cases of lung cancer. Sixty healthy controls (25 males and 35 females; 18–70 years old) were included in the study. The study was carried out with the consent of the university ethics committee.

Samples

Venous blood specimens were drawn from patients before therapy, and from controls, and placed into ethylenediaminetetracetic acid (EDTA) tubes and centrifuged at 3,000 rpm for 10 min at 4°C. Plasma and buffy coat were removed and the remaining erythrocytes were drawn from the bottom, washed three times in cold saline (9.0 g/L NaCl), and hemolyzed by the addition of an equal volume of ice-cold demineralized ultrapure water (MilliQ plus reagent grade; Millipore Corp., Bedford, MA) to yield a 50% hemolysate. Aliquots of the hemolysates (500 μ L) were prepared for the assay of SOD and GPX.

Assay of Erythrocyte SOD

Measurement of SOD activity was performed using reagents from Ransod (Randox Laboratories, Crumlin, UK) and is based on the method developed by McCord and Fridovich (5) coupling $O_2^{\cdot -}$ generators (xanthine and xanthine oxidase (XOD)) with an $O_2^{\cdot -}$ detector [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; I.N.T.]. Absorbance was monitored in a Beckman DU 7500 spectrophotometer (Beckman Instruments, Fullerton, CA) for 30 sec (for the initial reading (A_1)) after the addition of XOD (125 μ L) as the starting reagent and subsequently for 3 min for the final reading (A_2). The final reaction volume was 1 mL. All rates for the calibrators and the diluted samples were converted into percentages of the rate for the sample diluent (uninhibited) and subtracted from 100% to give a percentage of inhibition. The unit of activity of the assay is defined as the amount of SOD that inhibits the rate of formazan dye formation by 50%. The activity of SOD in units per liter divided by hemoglobin (Hb) in grams per liter gives the activity of SOD in units per gram of Hb. The precision of the assay was checked with Ransod control (Randox Laboratories, Crumlin, UK).

The intrabatch coefficient of variation (CV) after repeated analyses of the control ($n = 12$) was 4.7%, and the interbatch CV obtained by analyses of the same control material for 11 consecutive days was 5.8%

Assay of Erythrocyte GPX

Measurement of GPX was performed using the reagent from Ransel (Randox Laboratories) and is based on the method of Paglia and Valentine (6). GPX catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form (GSH) with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

Drabkin cyanide-ferricyanide solution, pH 7.0–7.4, was prepared by dissolving potassium ferricyanide (200 mg), potassium cyanide (50 mg), and potassium dihydrogen phosphate (140 mg) in distilled water, making the volume up to 1 L, and mixing after the addition of 0.5 mL of 250 g/L Brij 35.

For the GPX assay, hemolysates (50 μ L) were diluted with 1 mL of Ransel diluting agent (Ransel reagent; Randox) and incubated for 5 min, followed by the addition of 1 mL of the Drabkin reagent and mixing (total dilution of the hemolysates before assay = 1:82). The diluting agent reduces any GSSG present in the hemolysates to GSH because the cyanide in the Drabkin reagent rapidly inactivates GSSG. The cyanide in the Drabkin reagent also inhibits other peroxidases that may be present in human blood and prevents falsely high results.

One unit of GPX was defined as the amount of enzyme that catalyzed the transformation of 1 μ mol of NADPH per minute under the assay condition. The activity of GPX in units per liter divided by Hb concentration in grams per liter gives the activity of GPX in units per gram of Hb. Precision of the assay was checked with Ransel control (Randox Laboratories). The intrabatch precision obtained by repeated analyses of the control ($n = 12$) was 3.8%, and the interbatch precision obtained by analyses of the same control for 11 consecutive days was 5.3% (CV).

Statistical Analyses

Statistical analyses were performed by using SPSS 10.0 software (SPSS, Chicago, IL). The effects of disease, gender, age, and smoking on the data were determined using one-way analysis of variance (ANOVA) and least-squares difference (LSD) for a multiple-comparison test. The association between SOD and GPX in patients and controls was evaluated by linear

TABLE 1. Activities of erythrocyte SOD and GPX in controls and cancer patients*

Groups n	Controls 60	All 97	GIT 40	Breast 30	Others 27
SOD (U/g Hb)	1487±20	1182±34 <i>P</i> <0.0001	1179±54 <i>P</i> <0.0001	1216±66 <i>P</i> <0.0001	1142±55 <i>P</i> <0.0001
GPX (U/g Hb)	94.8±3.3	81.4±3.9 <i>P</i> =0.024	79.0±6.0 <i>P</i> =0.033	78.4±6.6 <i>P</i> =0.043	85.0±8.4 <i>P</i> =0.243

*The data are presented as mean±SEM. Statistical significance was considered at *P*<0.05.

regression. Also, the correlation between age and enzyme activities was assessed by least-square linear regression. Two-tailed *P* values are given throughout, and statistical significance is at *P*<0.05. Data are expressed as mean ± standard error of the mean (SEM).

RESULTS

The activities of erythrocyte SOD and GPX in the controls and the cancer patients are represented in Table 1. A highly significant decrease in the activity of erythrocyte SOD was found in all cancer sites (*P*<0.0001), GIT (*P*<0.0001), breast (*P*<0.0001), and others (*P*<0.0001) compared to the controls. The activity of erythrocyte GPX significantly decreased in all sites of cancer, GIT, and breast cancer in comparison with the controls (*P*=0.024, *P*=0.033, and *P*=0.043, respectively).

Table 2 shows the study characteristics of the cancer patients. There were no significant changes in data resulting from gender, smoking, or cancer duration. The correlation between age and the activities of SOD and GPX in cancer patients are presented in Table 3. There was a poor negative correlation between age and enzyme activities in hemolysates of patients and controls.

Figures 1 and 2 depict scatterplots and regression analyses on the relationship between the activities of erythrocyte SOD and GPX in cancer patients. There was no significant correlation between SOD and GPX activities in the healthy controls (*r*=−0.073, *P*=0.577) and patients (*r*=0.121, *P*=0.238).

DISCUSSION

The antioxidant enzyme assay is an essential component of the study of antioxidant status in health and in various diseases. The speculation regarding the long-term stability of red cell antioxidant enzymes (8) has been allayed by a recent study that demonstrated the prolonged stability of erythrocyte CuZn-SOD, GPX, and GR (9).

Hemoproteins such as oxyhemoglobin and oxymyoglobin are significant and important sources of reactive

TABLE 2. Study characteristics of the cancer patients*

Characteristics	n	SOD U/g Hb	GPX U/g Hb
Males	37	1122±57	77.7±5.7
Females	60	1217±41 <i>P</i> =0.188	83.6±5.2 <i>P</i> =0.451
Smokers	25	1135±72	90.0±8.1
Nonsmokers	72	1195±39 <i>P</i> =0.470	78.7±4.4 <i>P</i> =0.228
Cancer duration:			
≤ 3 months	47	1193±64	77.9±7.1
> 3 months	50	1087±54 <i>P</i> =0.213	91.7±6.8 <i>P</i> =0.163

*The data are presented as mean±SEM. Statistical significance was considered at *P*<0.05.

TABLE 3. Correlation between age and antioxidant enzyme activities in controls and cancer patients*

Subjects	n	Correlation coefficient (r)	<i>P</i> value
Controls: SOD vs age	60	−0.011	0.932
Patients: SOD vs age	97	−0.105	0.304
Controls: GPX vs age	60	−0.146	0.266
Patients: GPX vs age	97	−0.133	0.195

*Statistical significance was considered at *P*<0.05.

oxygen species (ROS) in biological systems. Their auto-oxidation has been shown to generate superoxide ($O_2^{\cdot -}$) through the transfer of electron from their ferrous component to oxygen (10). In health, erythrocytes are endowed with adequate antioxidant capacity in the form of antioxidant enzymes, CuZn-SOD, catalase, GPX, and GR, and other antioxidants that include vitamin E and glutathione to control the proliferation of ROS. However, the prevalence of ROS over antioxidants that occurs in oxidative stress produces oxidative tissue damage implicated in the pathogenesis of debilitating human diseases, including cancer (11). Ames and colleagues (12) previously proposed that cancer is a free radical disease that can produce oxidative stress. Assay

Fig. 1. Scatterplot and regression analysis showing the relationship between SOD and GPX activities in healthy controls.

Fig. 2. Scatterplot and regression analysis showing the relationship between SOD and GPX activities in cancer patients.

of antioxidant enzymes provides an indirect assessment of ROS generation in oxidative stress.

A number of previous case-control studies (13–17) examined the activities of antioxidant enzymes in various cancers. Two of these (13,14) assayed erythrocyte SOD and GPX activities, as was done in the current study. Significantly lower activities of erythrocyte SOD

and GPX compared to controls were found by Kumar and colleagues (13) in cases of postmenopausal breast cancer, and by Bewick and colleagues (14) in cases of malignant lymphoma. The remaining studies (15–17) evaluated only the activity of erythrocyte GPX in cancer. Significantly lower activities of erythrocyte GPX compared to controls were reported by Pawlowicz et al. (15) for cases of breast and GIT cancer, and by Zachara and colleagues (16) for lung cancer cases. A tendency for blood GPX activity to decrease as neoplasm formation and inanition progresses was observed by Robinson et al. (17). Our finding of a marked decrease in the activity of erythrocyte SOD in breast, GIT, and other cancers are in accord with the findings by Kumar et al. (13) and Bewick et al. (14). Regarding GPX, we noted that its depletion in activity compared to controls was not as significant as that of SOD (see Table 1). Previous studies (13–17) observed a significant decrease in activity of GPX for specific sites.

We observed a weak negative correlation between age and erythrocyte antioxidant enzyme activities in controls and cancer patients (see Table 3). Other variables such as gender, smoking, and cancer duration had no significant influence on SOD or GPX activity (see Table 1). Both SOD and GPX, along with other antioxidant enzymes such as catalase and glutathione reductase, are said to operate interdependently in the aqueous phase of cells. However, in the present study, judging from scatterplots and linear regression analyses, no association was seen between SOD and GPX in the controls (see Fig. 1), but there was a positive association in the cancer patients that was not statistically significant (see Fig. 2). The latter observation may suggest some degree of codependence between SOD and GPX activities in an oxidative stress condition such as cancer.

The much greater depletion in activity of SOD compared to GPX as demonstrated in this study may suggest a greater antioxidant burden for SOD than for GPX in cancer-generated oxidative stress. Although CuZn-SOD is more concentrated than GPX in erythrocytes, our data suggest that SOD may be more susceptible to cancer than GPX. There is evidence that SOD provides the first line of intracellular antioxidant protection against damage by ROS ($O_2^{\cdot-}$) (18), a role that may be more exhausting than the inactivation of H_2O_2 , shared by GPX and catalase. Because catalase is more concentrated than GPX in erythrocyte (19), if the concentration of H_2O_2 is raised (as is the case in oxidative stress), catalase becomes more important than GPX, which efficiently reduces H_2O_2 at a low concentration. In general, our data suggest that depleted antioxidant activity in cancer is probably related to DNA damage in oxidative stress.

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