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Lipid Peroxidation and Antioxidant Enzyme Activities in Cancerous Bladder Tissue and Their Relation with Bacterial Infection: A Controlled Clinical Study

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It is well known that antioxidants and reactive oxygen species play an important role in carcinogenesis. In this sudy, we attempted to evaluate antioxidant enzyme activities and lipid peroxidation levels in cancerous bladder tissue and to determine their relationship with bacterial infection. Bacterial culture was made from all urine samples using Blood and Eosin Methylene Blue agars for checking the presence of bacterial infections. We measured thiobarbituric acid reactive substances (TBARs) and activities of xanthine oxidase (XO), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase (CAT) in cancerous tissues of 25 bladder cancer patients, in noncancerous adjacent bladder tissues of 13 out of these 25 patients, and in control bladder tissues of 15 patients with a non-neoplastic genitourinary disease. TBARs levels increased and XO, SOD, GSH-PX, and CAT activities decreased significantly in cancerous bladder tissues. TBARS, XO, and SOD levels were not significantly different between noncancerous adjacent tissue and control bladder tissue. Statistically significantly lower GSH-PX and higher CAT activities were observed in noncancerous adjacent bladder tissue compared with cancerous tissue. GSH-PX level of tumor tissue was correlated significantly with tumor grade ($r = -0.425$, $P = 0.034$). Results suggested that pathway activity of free radicals were accelerated in the cancerous human bladder tissues via increased TBARs levels and decreased enzyme activities of XO, SOD, GSH-PX, and CAT, which implicated a severe exposure of cancerous tissues to oxidative stress. J. Clin. Lab. Anal. 24:25–30, 2010.

Key words: bladder cancer; xanthine oxidase; superoxide dismutase; glutathione peroxidase; catalase; thiobarbituric acid reactive substances

INTRODUCTION

Modification of the activity of antioxidant enzymes that play essential roles in the defense mechanisms of cells against oxygen toxicity have been observed in various cells of bladder cancers (1). The importance of antioxidants and reactive oxygen species in carcinogenesis was considered in experimental studies in vivo (2). One of the most important antioxidant enzymes is superoxide dismutase (SOD) catalyzing dismutation of the superoxide anion into hydrogen peroxide (H_2O_2), which has been removed by catalase (CAT) and glutathione peroxidase (GSH-PX) (3,4). The GSH-PX contains selenium (essential for catalytic function) at its active site, as does a similar enzyme that can remove lipid hydroperoxides from membranes (5). Under some circumstances, these endogenous antioxidative defenses are likely to be perturbed as a result of overproduction of oxygen radicals, inactivation of detoxification systems (6), and failure to replenish these antioxidants in tissues adequately. It has been demonstrated in numerous studies that ROS are directly involved in oxidative damage of cellular macromolecules such as lipids,

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proteins, and nucleic acids in tissues (7,8). Xanthine oxidase (XO) is the last enzyme functioning in the purine metabolism, which converts hypoxanthine and xanthine to uric acid with production of superoxide radical. In several studies, changed XO activities were observed in cancerous tissues (9,10).

Despite scavenge activity of all these antioxidants, some free radicals still escape to do damage. Thus DNA, proteins, and lipids undergo constant "Oxidative damage." Free radical-damaged proteins are degraded. End-products of lipid peroxidation and free radicals which attack on urate are present *in vivo* (2). The systemic oxidative stress was evaluated by the estimation of the total antioxidant capacity and/or antioxidant enzyme activities individually, which can respond to increased oxidative stress by compensatory increase in their activities (11). Antioxidative enzymes exist in cells to protect against the toxic effects (12).

On the light of these facts, this study was designed to investigate the role of activities of key antioxidant enzymes, such as SOD, CAT, GSH-PX, XO, functioning in free radical metabolism, and lipid peroxide status on the growth of human malignant bladder tumors by determining their activities in tumor tissue in patients with bladder cancer as compared with the values in noncancerous adjacent bladder tissue of the same cancer patients and with those in bladder tissue of control patients with a non-neoplastic genitourinary disease.

MATERIALS AND METHODS

Subjects

This study was approved by Medical Research Ethics Committee of Inonu University. Following the written informed consents of the patients, study was carried out at Inonu University, Urology and Biochemistry Departments between June 2004 and October 2007.

Tumor group was composed of 25 patients (1 female, 24 males) with proven bladder cancer documented by pathologic examination of resection specimens obtained during transurethral resection. Patients who preoperatively received intravesical BCG instillation therapy, radiotherapy, or chemotherapy for their bladder carcinoma diseases were not included in the study. The histological grading and staging for tumor tissue samples were determined according to generally accepted criteria (13,14). Fifteen patients, who underwent a transurethral procedure (cystoscopy, internal uretrotomy, transurethral prostatectomy, transurethral bladder neck resection, or cystolithotripsy) for a non-neoplastic genitourinary disease and did not have any documented cancer, served as controls.

Tissue samples for SOD, CAT, GSH-PX, XO, and MDA determination were preserved at $-80^{\circ}C$ till processing. Tumor tissue specimens were obtained from all cancer patients. Normal bladder tissue specimens, which served as controls, were obtained from the nonmalignant adjacent tissue in 13 cancer patients and from 15 control patients. Tissue samples were rapidly perfused with ice-cold saline (0.9% NaCl w/v). At working site, they were codified and transported to the laboratory where they would be processed. They were washed with distilled water, blotted, dissected out, and folded with aluminum foil and stored at $-80^{\circ}C$ till analyzing.

Antioxidant activities in cell-free systems

XO activity

It was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm, according to Prajda and Weber's method (15). A calibration curve was constructed by using 10–50 mU/ml concentrations of standard XO solutions (Sigma X-1875 SIGMA-ALDRICH Chemie GmbH Export Department Eshenstrafÿe 5, Taufkirchen, Germany). One unit of activity was defined as 1 μmol of uric acid formed per minute at $37^{\circ}C$, pH 7.5, expressed in units per liter (U/l) and calculated as mean $\pm$ standard deviation.

SOD actitity

It was measured spectrophometrically as described by Sun et al. (16). Briefly, xanthine-xanthine oxidase was utilized to generate a superoxide flux. Reduction of nitrobluetetrazolium (NBT) by superoxide anion to blue formazon was determined at 560 nm. One unit of enzyme activity was defined as the amount of protein causing 50% inhibition in NBT reduction by superoxide.

GSH-Px

The activity of GSH-PX in serum was measured spectrophometrically as described by Paglia and Valentine (17). The enzyme reaction was initiated by the addition of H_2O_2 and the rate of NADPH oxidation was followed at 340 nm. One unit of GSH-PX was expressed as the amount of enzyme that oxidizes one micromole NADPH per minute.

CAT

The catalytic activity of CAT was determined as described by Aebi (18). The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. One unit of enzyme was defined as one micromole H_2O_2 utilized per minute.

Bacteriological Examination

Urine samples were collected from all individuals in sterile bottles and cultured on blood and Eosin Methylene Blue agar to determine whether urinary infection was present or not.

Protein Determination

All protein determinations in samples were done according to Lowry et al. using bovine serum albumin as standard (19). A Shimadzu 1601 UV-VIS spectrophotometer was employed for protein determination and enzyme assays.

Thiobarbituric acid reactive substances (TBARs) Measurements

The fluorescence spectrophotometric method described by Wasowich et al. was followed using Hitachi Model 4010 spectrofluorometer (20). The amount of colored complex obtained by the reaction of MDA and thiobarbituric acid was determined employing 525 and 547 nm for excitation and emission, respectively. The concentration of MDA was described as micromoles per liter of serum.

Statistical Analysis

Values represent means $\pm$ standard deviation (SD) unless otherwise stated. Analysis was made with SPSS for Windows (version 10.0). The distributions of all parameters were tested using Kolmogorov–Smirnov's goodness of fit test and it was found to be normal. All statistical analysis methods used were indicated within the text and tables.

RESULTS

Mean age was 63.6 ± 12.8 years (range: 30–84) in tumor group and 63.7 ± 8.9 years (range: 43–94) in control group ($P = 0.968$, independent samples t test). Complaints in tumor group at presentation were macroscopic hematuria in 16 patients, infravesical obstruction symptoms in 2, and hematuria plus infravesical obstruction in another 2 patients. The remaining 5 patients were admitted to the hospital for cystoscopic surveillance of previously treated bladder transitional cell carcinoma (TCC) only by resection.

In tumor group analysis of the urine taken immediately before the operation showed microscopic and macroscopic hematuria in 9 and 12 patients, respectively. Examination was normal in the remaining 4.

Preoperative ultrasonography and computerized tomography revealed unilateral and bilateral ureterohydronephrosis associated with the obstruction of ureter orifices by tumor in six and two patients, respectively. However, creatinine and blood urea nitrogen were increased in only one patient.

Respectively, urinary infection incidences in cancer and control patients were 36% (9/25) and 34.1% (14/41) at preoperative period, difference being statistically insignificant ($P = 0.878$, independent samples t test).

Pathological examination of tumor specimens defined squamous cell carcinoma in 1 (pT1G2), adenocarcinoma in 1 (pT4aG3), and TCC in 23 patients (5 pTaG1, 2 pTaG2, 7 pT1G2, 1 pT2aG1, 3 pT2bG2, 4 pT2bG3, and 1 pT4aG2).

As regard to urinary bacterial infection, there were no statistically significant differences in SOD, GSH-Px, XO, and MDA means of tumor tissue between the patients with and without bacterial infection of tumor group (Table 1). Only CAT was statistically greater in the patients with no infection than in those with infection ($P = 0.015$). In 13 control patients of tumor group, no significant differences were found in SOD, GSH-Px, XO, CAT, and TBARs means of nonmalignant adjacent bladder tissues of patients with and without infection. Similarly, means of all tissue parameters measured were also statistically similar between the patients of control group with no documented cancer as independent from the presence of urinary infection.

TABLE 1. Regarding to the Presence of Urinary Infection, TBARS Levels and XO, SOD, GSH-PX, CAT Enzyme Activities in Tumoural and Nontumoural Bladder Tissues

	Tumoural bladder tissue			Nonmalignant adjacent bladder tissue			Control bladder tissue		
	Inf+n = 9 pts.	Inf–n = 16 pts.	P[a]	Inf+n = 5 pts.	Inf–n = 8 pts.	P[b]	Inf+n = 6 pts.	Inf–n = 9 pts.	P[b]
TBARS (nmol/mg)	256.7 ± 22.21	256.9 ± 36.18	0.989	119.9 ± 8.58	126.9 ± 7.46	0.305	124.7 ± 5.79	122.6 ± 6.77	0.637
XO (mIU/mg)	3.12 ± 0.08	3.18 ± 0.10	0.158	4.12 ± 0.05	3.97 ± 0.17	0.142	4.16 ± 0.2	4.066 ± 0.21	0.516
SOD (U/mg)	0.071 ± 0.007	0.072 ± 0.005	0.868	0.133 ± 0.007	0.141 ± 0.012	0.212	0.14 ± 0.016	0.14 ± 0.013	0.768
GSH-PX (IU/mg)	0.235 ± 0.011	0.231 ± 0.008	0.245	0.504 ± 0.014	0.491 ± 0.014	0.057	0.54 ± 0.028	0.512 ± 0.038	0.059
CAT (IU/mg)	5.60 ± 1.03	6.57 ± 0.80	0.015	14.73 ± 1.194	13.77 ± 1.541	0.213	11.12 ± 4.022	12.01 ± 3.65	1.000

[a]Independent samples test.
[b]Mann-Whitney U test.

TABLE 2. TBARS Levels and XO, SOD, GSH-PX, CAT Enzyme Activities in the Groups of Nonmalignant adjacent Bladder Tissue (A; no: 13), Cancerous Bladder Tissue (B; no: 25), and Control Bladder Tissue (C; no: 15) (Mean ± SD)

	A	B	C	P^a (A−B)	P^b (B−C)	P^b (A−C)
TBARS (nmol/mg)	124.19 ± 8.32	256.79 ± 31.35	123.22 ± 6.22	<0.0001	<0.0001	0.727
XO (mIU/mg)	4.026 ± 0.15	3.158 ± 0.097	4.103 ± 0.203	<0.0001	<0.0001	0.259
SOD (U/mg)	0.138 ± 0.0105	0.0715 ± 0.0056	0.140 ± 0.0134	<0.0001	<0.0001	0.715
GSH-PX (IU/mg)	0.496 ± 0.015	0.232 ± 0.0091	0.523 ± 0.034	<0.0001	<0.0001	0.010
CAT (IU/mg)	14.139 ± 1.448	6.220 ± 0.991	11.651 ± 3.684	<0.0001	<0.0001	0.026

[a]Paired samples test.
[b]Independent samples test.

The mean TBARs, XO, SOD, GSH-Px, CAT activities in tumor tissue and nontumoral adjacent bladder tissue from the patients with bladder cancer and in bladder tissue from control patients are given in Table 2. There was statistically very significant increase in TBARs level and significant decrease in XO, SOD, GSH-Px, and CAT activities in tumor tissue compared with the values in nontumoral adjacent bladder tissue and in control bladder tissue. When comparison was made between the nontumoral adjacent bladder tissue of tumor patients and control bladder tissue, statistically significantly lower GSH-Px activity and higher CAT activity were observed in nontumoral adjacent bladder tissue.

There were no significant correlations between age of tumor patients and grade and stage of the tumor. As expected, histological grade of the tumor correlated strongly and positively with pathologic stage of the tumor ($P = 0.001$) and with clinical stage ($P = 0.002$). GSH-PX level of tumor tissue correlated significantly with tumor grade (correlation coefficient $r = -0.425$, $P = 0.034$). No other correlations were detected between the tumoral or nontumoral tissue levels of measured parameters and age, tumor grade or stage.

DISCUSSION

Cancer is the largest single cause of death in both men and women. Reactive oxygen or nitrogen species (RONS) are produced during tissue damage due, at least in part, to the activation of XO (21,22). XO degradation leads to the formation of H_2O_2 and hydroxyl radicals (22). Although both O_2^- and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by the OH^- radical, which is generated by the reaction between O_2^- and H_2O_2 in the presence of transition metal ions (23). Decreased XO activities in bladder cancer tissue in our study suggested that oxidative stress might be increased in course of the disease (24,25). The burst of XO-mediated free oxygen radical generation in the cancerous tissue can be triggered by a large increase in substrate formation, which occurs secondary to the rapid turnover of adenine nucleotides during cancer process (8,24,25). The generation of oxygen radicals is probably responsible for the disturbance of cell membranes by lipid peroxidation and leads to tissue and/or organ damage (26,27). Cellular mechanisms and external factors involved in the production of oxidative stress include the inflammatory response, free radical leak from mitochondria, auto-oxidation of catecholamines, XO activation, prooxidant activities of toxins such as carbon tetrachloride, and exposure to ionizing radiation (28). Aerobic cells are endowed with extensive antioxidant defense mechanisms including both low molecular weight scavengers, reduced enzymatic systems, such as SOD, CAT, GSH-Px, which counteract the damaging effects of reactive oxygen species (29). This study indicated that cancer tissue samples could be used as a test material to study oxidative stress by evaluating their XO enzyme activities (12). To our knowledge, the results of our study supported with the results obtained before by the other researchers (26,30). In animals study the repercussions on the antioxidant and lipid peroxide status of the growth of human malignant tumors xenografted into athymic mice was evaluated (27). It would appear that an oxygen-mediated stress exists in the animal bearing an implanted tumor compared with the control group, and that tumoral tissue itself is able to induce an oxidative stress into its host (28,29). All this leads to a disturbance of the antioxidant defense system. We studied antioxidant enzymes SOD, GSH-Px, and CAT in tissues and found that all these enzymes activities were significantly lower compared with the controls. As it is known the primary role of SOD is to lower the steady state level of superoxide anion (O_2^-). Decreased levels of SOD, therefore, causes increased levels of O_2^- that creates oxidative stress (30,31). Decreased CAT and GSH-Px levels also indicate a state of oxidative stress via accumulation of H_2O_2, which depresses SOD activity in return (32). Lipid peroxidation is one of the best-known manifestations of oxidative cell injury. A major final product of lipid peroxidation, TBARs, found in tissue can serve in

evaluating the extent of oxidative damage. Kidney disease had elevated TBARs concentrations in tissue which reflect the extent of lipid peroxidation (33). In this study, fluorescence spectroscopy was used for determination of TBARs. This method has certain limitations and TBARS observed may be slightly higher than that other investigators who may use more specific sophisticated high performance liquid chromatography assay.

Pavlova et al. reported increased TBARs and decreased of SOD, GSH-Px and CAT activities in infectious patients. It is generally accepted that uropathogenic bacteria, such as *Escherichia coli*, may exist during urinary tract infection (32,34). We evaluate in this study the dynamics of oxidative stress in the tissue of bladder cancer and investigate lower urinary tract infections and antioxidant conditions. There were no statistically significant differences in SOD, GSH-Px, XO, and TBARs means of tumor tissue and of nonmalignant adjacent bladder tissues of patients with and without urinary infection in tumor group (Table 1). Only CAT was statistically greater in the patients with no infection than those with infection. In 13 control patients of tumor group, no significant differences were found in SOD, GSH-Px, XO, and CAT (Table 1). Similarly, means of all tissue parameters measured were also statistically similar between the patients of control group with no documented cancer as independent from the presence of urinary infection. Several studies have concluded that increased TBARs level and decreased SOD, GSH-Px, and XO in infectious tumor tissue as contrary to our results (29,30,34).

The controversy found between the studies indicated that enzyme metabolism in cancer might show great differences depending on cancerous tissues studied. The mechanisms put forward to explain enzyme changes in carcinogenesis might be specific only for the analyzed material. These different findings also display difficulties encountered in the explanation of enzymatic mechanisms in cancer processes. The diverse results might arise from the fact that enzyme activities were studied in different kinds of tissue and under different conditions (35,36). There might be several reasons for the differences observed between the cancerous and noncancerous tissues (36). They could result from the carcinogenesis process itself (36). We find a correlation between TBARs and CAT activities of tumor tissue. We could not find a correlation between TBARs, SOD, GSH-Px, XO, and CAT activities of tumor tissue and tumor grade or stage of tumor tissue. We also find negative correlation between tumor grade or stage and tumor tissue GSH-Px levels, a statistical evaluation with more patients would be necessary (37). An inverse correlation has been defined between the GSH-Px levels in the presence and the differentiation of the tumor in human bladder and human colorectal carcinoma cells (37). These findings may reflect the functioning of both generalized and localized defense systems together against the growth and progression of bladder cancer cells. Localized antioxidant enzyme defense systems are seen effective in the presence of tumor. This could be explained by the small patient populations in different tumor grade groups.

Definitive conclusions need further evaluation with longer observation periods and larger numbers of patients. These data could be helpful for the optimization of complex and effective antioxidant therapy of patients with bladder cancer disease.

In conclusion, increased TBARs and XO activities and decreased SOD, GSH-Px, and CAT activities in bladder cancer tissue might be a potentially important finding as an additional diagnostic for bladder cancer. Further investigations in a larger cohort of patients with bladder cancer are needed to enlighten the possible diagnostic role of increased TBARs levels and decreased XO, SOD, GSH-Px, and CAT activities in bladder cancer tissue.

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